



(86) **Date de dépôt PCT/PCT Filing Date:** 2009/07/31
(87) **Date publication PCT/PCT Publication Date:** 2010/02/04
(45) **Date de délivrance/Issue Date:** 2018/08/21
(85) **Entrée phase nationale/National Entry:** 2011/01/31
(86) **N° demande PCT/PCT Application No.:** US 2009/052436
(87) **N° publication PCT/PCT Publication No.:** 2010/014920
(30) **Priorités/Priorities:** 2008/07/31 (US61/085,175);
2009/07/30 (US12/512,585)

(51) **Cl.Int./Int.Cl. C12Q 1/6809** (2018.01),
C12Q 1/68 (2018.01), **C12Q 1/6827** (2018.01),
G06F 19/20 (2011.01)

(72) **Inventeurs/Inventors:**
DIEHL, FRANK, DE;
DIAZ, LUIS, US;
KINZLER, KENNETH W., US;
VOGELSTEIN, BERT, US;
SCHMIDT, KERSTIN, US

(73) **Propriétaire/Owner:**
THE JOHNS HOPKINS UNIVERSITY, US

(74) **Agent:** TORYS LLP

(54) **Titre : ADN MUTANT CIRCULANT POUR EVALUER UNE DYNAMIQUE DE TUMEUR**
(54) **Title: CIRCULATING MUTANT DNA TO ASSESS TUMOR DYNAMICS**

(57) **Abrégé/Abstract:**

DNA containing somatic mutations is highly tumor specific and thus, in theory, can provide optimum markers. However, the number of circulating mutant gene fragments is small compared to the number of normal circulating DNA fragments, making it difficult to detect and quantify them with the sensitivity required for meaningful clinical use. We apply a highly sensitive approach to quantify circulating tumor DNA (ctDNA) in body samples of patients. Measurements of ctDNA can be used to reliably monitor tumor dynamics in subjects with cancer, especially those who are undergoing surgery or chemotherapy. This personalized genetic approach can be generally applied.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau(43) International Publication Date
4 February 2010 (04.02.2010)(10) International Publication Number
WO 2010/014920 A1(51) International Patent Classification:
C12Q 1/68 (2006.01)(21) International Application Number:
PCT/US2009/052436(22) International Filing Date:
31 July 2009 (31.07.2009)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/085,175 31 July 2008 (31.07.2008) US
12/512,585 30 July 2009 (30.07.2009) US(71) Applicant (for all designated States except US): **THE JOHNS HOPKINS UNIVERSITY** [US/US]; 5th Floor, 100 N. Charles Street, Baltimore, MD 21201 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **DIEHL, Frank** [DE/DE]; Osterweg 9, 26419 Schortens (DE). **DIAZ, Luis** [US/US]; 5943 Meadow Rose, Elkridge, MD 21075 (US). **KINZLER, Kenneth, W.** [US/US]; 1403 Halkirk Way, Baltimore, MD 21015 (US). **VOGELSTEIN, Bert** [US/US]; 3700 Breton Way, Baltimore, MD 21208 (US). **SCHMIDT, Kerstin** [DE/US]; 1908 Convent Place, Apt. 13, Nashville, TN 37212 (US).(74) Agent: **KAGAN, Sarah, A.**; Banner & Witcoff, Ltd., 1100 13th Street, N.w., Suite 1200, Washington, DC 20005-4051 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— with international search report (Art. 21(3))

(54) Title: CIRCULATING MUTANT DNA TO ASSESS TUMOR DYNAMICS

(57) Abstract: DNA containing somatic mutations is highly tumor specific and thus, in theory, can provide optimum markers. However, the number of circulating mutant gene fragments is small compared to the number of normal circulating DNA fragments, making it difficult to detect and quantify them with the sensitivity required for meaningful clinical use. We apply a highly sensitive approach to quantify circulating tumor DNA (ctDNA) in body samples of patients. Measurements of ctDNA can be used to reliably monitor tumor dynamics in subjects with cancer, especially those who are undergoing surgery or chemotherapy. This personalized genetic approach can be generally applied.



WO 2010/014920 A1

CIRCULATING MUTANT DNA TO ASSESS TUMOR DYNAMICS

[01] This application was made using US government funding. The US government retains certain rights in the invention according to the terms of CA43460, CA62924, and CA 57345.

TECHNICAL FIELD OF THE INVENTION

[02] This invention is related to the area of cancer. In particular, it relates to cancer diagnosis, prognosis, therapeutics, and monitoring.

BACKGROUND OF THE INVENTION

[03] Cancers arise through the sequential alteration of genes that control cell growth. In solid tumors such as those of the colon or breast, it has been shown that, on average, approximately 80 genes harbor subtle mutations that are present in virtually every tumor cell but are not present in normal cells¹. These somatic mutations thereby have the potential to serve as highly specific biomarkers. They are, in theory, much more specific indicators of neoplasia than any other biomarker yet described. One challenge for modern cancer research is therefore to exploit somatic mutations as tools to improve the detection of disease and, ultimately, to positively affect individual outcomes.

[04] Tumor cells can often be found in the circulation of individuals with advanced cancers^{2,3}. It has been shown that tumor-derived mutant DNA can also be detected in the cell-free fraction of the blood of people with cancer⁴⁻⁶. Most of this mutant DNA is not derived from circulating tumor cells⁴⁻⁶ and, in light of the specificity of mutations, raises the possibility that the circulating mutant DNA fragments themselves can be used to track disease. However, the reliable detection of such mutant DNA fragments is challenging⁷.

In particular, the circulating mutant DNA represents only a tiny fraction of the total circulating DNA, sometimes less than 0.01%⁸.

- [05] In the current study, we developed modifications of a technique called BEAMing (Beads, Emulsion, Amplification and Magnetics)^{8,9} to quantify ctDNA in serially collected plasma samples from subjects with colorectal cancers. We were interested in determining whether such measurements provided information about the dynamics of tumor burden in these subjects during the course of their disease.
- [06] There is a continuing need in the art for ways to better determine which patients will experience relapses of their cancer and which will not.

SUMMARY OF THE INVENTION

- [07] According to one embodiment of the invention, a method is provided to monitor tumor burden. Number of copies of DNA fragments in a test sample of a cancer patient is measured. The DNA fragments have a mutation that is present in tumor tissue of the patient but not in normal tissue of the patient. The number of copies is an index of the tumor burden in the patient.
- [08] According to another embodiment, a method is provided for performing DNA analysis. The following steps are involved:
- a. amplifying a template DNA analyte with a first primer set and a second nested primer set, wherein one member of the second nested primer set comprises a 5' sequence 5'-tccgcgaaattaatacgac (SEQ ID NO: 1), wherein the amplifying employs a high fidelity DNA polymerase;
 - b. amplifying in an aqueous medium the amplified template using a third primer set, wherein one member of the third primer set comprises a 5' sequence 5'-tccgcgaaattaatacgac (SEQ ID NO: 1), and a second member of the third primer set comprises a 5' sequence 5'-gctggagctctgcagcta (SEQ ID NO: 2), and

- streptavidin beads coated with 5'-tcccgcgaaattaatacgac (SEQ ID NO: 1)
oligonucleotide;
- c. preparing an water-in-oil emulsion using the aqueous medium as the aqueous phase and an oil/emulsifier mixture;
thermally cycling the emulsion to amplify the template on the bead;
breaking the emulsions using detergent and removing the oil phase;
 - d. forming a mixture of the amplified templates on the bead with a mutation-specific probe, a corresponding wild-type probe, and an amplicon-specific probe that is complementary to a portion of the template distinct from the mutation-specific probe and the corresponding wild-type probe, wherein each of the probes is fluorescently-labeled and each of the probes has a distinct emission spectrum;
 - e. thermally denaturing amplified templates in the mixture and cooling the mixture in the presence of tetramethyl ammonium chloride (TMAC) to hybridize the probes to the templates;
 - f. analyzing the hybridized templates using flow cytometry to detect the amount of each of the fluorescently-labeled probes hybridized to amplified templates on the beads.

[09] These and other embodiments which will be apparent to those of skill in the art upon reading the specification provide the art with methods which are useful for cancer patient management and monitoring.

BRIEF DESCRIPTION OF THE DRAWINGS

[10] Figs. 1A-1C show measurement of ctDNA. Fig. 1B depicts conventional Sanger sequencing of DNA derived from the subject's tumor, representing the first step of the analysis. The approach for quantifying tumor-derived DNA in plasma samples is shown in Fig. 1C. Real-time PCR is used to measure the total number of DNA fragments in the plasma, whereas BEAMing measures the ratio of mutant to wild-type fragments labeled with Cy5 and Cy3 are fluorescent probes.

- [11] Fig. 2A to 2D shows representative flow cytometric data obtained from BEAMing. The four graphs illustrate the data obtained from subject 6 (*APC* G4189T) at different time points during treatment. The northwest quadrant dots and southeast quadrant dots represent beads bound to wild-type and mutant fragments, respectively. The northeast quadrant dots represent beads bound to both wild-type and mutant fragments resulting from their inclusion in an emulsion microdroplet that contained both wild-type and mutant DNA templates¹⁵. Numbers in each quadrant represent absolute counts of beads for each population measured. (Fig. 2A) Before surgery, the fraction of mutant DNA fragments was 13.4%. (Fig. 2B) After surgery (day 3), the fraction of mutant DNA fragments dropped to 0.015%. (Fig. 2C) After surgery (day 48), the fraction of mutant DNA fragments increased to 0.11%, suggesting disease recurrence. (Fig. 2D) On day 244, the subject had progressive disease and the fraction mutant DNA fragments increased further to 0.66%.
- [12] Figs. 3A-3B show recurrence-free survival, as detected by ctDNA and CEA. (Fig. 3A) The difference in recurrence-free survival in subjects with detectable versus undetectable post-operative ctDNA levels ($P = 0.006$ by Mantel-Cox log-rank test). (Fig. 3B) The difference in recurrence-free survival in subjects with detectable versus undetectable post-operative CEA levels ($P = 0.03$ by Mantel-Cox log-rank test).
- [13] Figs. 4A-4C show comparison of ctDNA, CEA and imaging dynamics in individual study subjects. For each subject, the top, middle, and bottom graphs represent ctDNA level, tumor volume as assessed by imaging, and CEA level. The horizontal lines represent the upper bound of the normal levels: one mutant DNA fragment per sample for ctDNA levels, 0.0 cm for tumor diameter, and 5.0 ng ml^{-1} for CEA abundance. Fig. 4C: Patient 8 had a sigmoid adenocarcinoma and solitary metastases in both hepatic lobes. The subject underwent a sigmoidectomy and left lateral hepatic sectorectomy (Surgery 1). A right-sided liver metastasis was left in place while the subject was treated with systemic chemotherapy (Chemotherapy 1). On day 120, a right hepatectomy was performed (Surgery 2). After surgery, the subject was treated for 4 months with systemic chemotherapy (Chemotherapy 2). Fig. 4B: Patient 11 had a sigmoid adenocarcinoma and

two liver metastases that were treated with systemic chemotherapy before surgery (Chemotherapy 1). The subject underwent a sigmoid colectomy, left hepatic lobectomy and RFA of a solitary right hepatic lesion (Surgery 1). Imaging studies at 2 months showed recurrence in the liver, and the subject underwent a right hepatectomy (Surgery 2). Given the high risk of recurrence, chemotherapy was reinitiated (Chemotherapy 2). At 8 months, imaging showed three recurrent liver lesions and a suspicious celiac lymph node. The subject underwent RFA of these lesions and resection of the celiac node (Surgery 3). After surgery, the subject received additional chemotherapy (Chemotherapy 3); however, later imaging revealed multiple pulmonary metastases. Fig. 4A: Patient 5 underwent a left hepatectomy for recurrent disease at the time of entry into the study (day zero). Except for a questionable lung nodule in the left upper lobe, there was no evidence of disease immediately after surgery. Fifteen months later, disease recurrence was noted, with lesions found in both liver and lung.

- [14] Fig. 5A-5B shows a schematic of the BEAMing-based approach for detecting mutant DNA in stool samples from patients with colorectal cancer. (Fig. 5 A) depicts the stages in the process, starting with total fecal DNA. Step 1 represents the results of sequence-specific capture of mutant and wild-type single-stranded DNA molecules. After PCR-mediated amplification of gene fragments encompassing the queried mutation sites, the DNA is mixed with magnetic beads (spheres) that are bound to oligonucleotides (spikes on the spheres) complementary to sequences in the PCR products (step 2). In Step 3, this mixture is segregated into billions of microcompartments in a water-in-oil emulsion. A small portion of these compartments contain a single bead and a single DNA template molecule, while the great majority of compartments contain neither (such as the empty bubble in the middle). When PCR is performed on these emulsions in Step 4, individual DNA fragments are amplified within the microcompartments that contain them and become covalently bound to the surface of the bead. The resultant beads are coated with tens of thousands of copies of identical DNA fragments. In Step 5, beads are recovered from the emulsion and the sequence of the bound DNA is deciphered by allele-specific hybridization (ASH) as depicted in panel (Fig. 5 B). (Fig. 5 B) DNA amplified on

magnetic microbeads by BEAMing is initially denatured to remove the non-covalently bound DNA strand. Differently labeled fluorescent probes are hybridized to the complementary target DNA covalently bound to the beads. Flow cytometry is then used to individually count beads, thereby determining the ratio of mutant to wild-type fragments originally present in the stool or plasma sample.

- [15] Figs. 6A-6D show scatter plot of beads analyzed by flow cytometry. BEAMing assay for APC C4132T mutation using normal lymphocyte DNA (Fig. 6A) or stool DNA from patient 4 (Fig. 6B). For lymphocyte DNA the total number of beads analyzed (all quadrants) was 253,723 with no bead containing mutant DNA (southeast quadrant, *i.e.*, quadrant 4). The total number of beads analyzed for patient 4 was 192,513, of which 747 were mutant. (Fig. 6C) BEAMing assay for KRAS G38A using stool DNA from patient 12, whose tumor did not contain this mutation. Five mutant beads were present among 305,449 analyzed beads, which were introduced by the DNA polymerase used for the initial amplification and scored as negative. (Fig. 6D) Assay of stool DNA from patient 7 whose tumor did contain a KRAS G38A. A total of 333,630 beads were analyzed, of which 685 beads were mutant.
- [16] Fig. 7A-7D shows quality and quantity of normal and mutant DNA isolated from stool of patients with CRC. (Fig. 7A) Schematic of experimental design. Stool DNA was amplified with differently sized primer pairs that encompass a patient-specific DNA mutation. Real-time PCR was used to determine the total number of stool DNA fragments obtained for each amplicon size. These amplified fragments were subsequently analyzed by BEAMing to determine the number of normal Fig. 7 (B) and mutant (Fig. 7C) DNA fragments as well as the fraction of mutant to normal molecules Fig. 7 (D) present in the feces (-●- Patient 2, -■- Patient 4, -▲- Patient 7, -▼- Patient 14)

- [17] Fig. 8 shows mutations in fecal DNA and TNM stage. The horizontal bar shows the median fraction of mutant DNA. The whiskers represent the minimum and maximum values that were found for each indicated stage.
- [18] Fig. 9. Primers used for amplification of stool and plasma DNA. Pairs of forward and reverse primers (SEQ ID NOs: 10-53); Tag 1 (SEQ ID NO: 54); Tag 2 (SEQ ID NO: 55).
- [19] Fig. 10A-10B. Probe sequences for allele-specific hybridization, SEQ ID NOs: 56-154.
- [20] Fig. 11. Primers used for fragment sizing, SEQ ID NOs: 155-182; Tag 1, SEQ ID NO: 183.
- [21] Fig. 12 . Summary of sensitivities
- [22] Fig. 13. Mutations in tumor and stool DNA determined by SBE and Sequencing
- [23] Fig. 14 shows ctDNA clearance after resection. The y-axis represents the level of ctDNA in the plasma of patient 9. The x-axis represents the time from resection, with zero as the time of tumor removal. To calculate the half-life, a curve fit ($f(t) = a - \lambda t$) based on the Marquardt-Levenberg algorithm was performed, yielding a half-life of 114 min.
- [24] Fig. 15 shows total DNA fragments in plasma prior to and after surgery. The Wisker box plot shows the total number of DNA fragments in 2 ml plasma, estimated by real-time PCR at baseline (day 0), post-surgery (day 1), day of discharge (days 2-5), and at the 1st follow-up (days 13-56).
- [25] Figs. 16A-1 to 16E-3 show molecular biologic, clinical, and radiologic data on all patients in addition to those shown in Fig. 4.
- [26] Fig. 17 shows a comparison between plasma CEA and ctDNA levels in the same plasma samples. A partial residual plot comparing CEA and ctDNA levels, corrected for individual clustering, is shown. All patients' CEA and ctDNA values were used for this

comparison. There was a modest overall correlation between CEA levels and ctDNA after correcting for clustering within patients ($r^2 = 0/2$, $P < 0.001$).

- [27] Fig. 18 shows plasma collection time-line
- [28] Fig. 19 lists the characteristics of the eighteen colorectal cancer patients evaluated.
- [29] Fig. 20 shows the 26 amplicons that were analyzed by direct sequencing. Forward primers (SEQ ID NO: 184-235). Reverse primers (SEQ ID NO: 236-287). Tag 1 (SEQ ID NO: 288). Tag 2 (SEQ ID NO: 289).
- [30] Fig. 21 shows the primers used for BEAMing each test of amplicons. Forward primers (SEQ ID NO: 290-305). Reverse primers (SEQ ID NO: 306-321). Tag 1 (SEQ ID NO: 322). Tag 2 (SEQ ID NO: 323).
- [31] Fig. 22 shows the probes used for each test of amplicons (SEQ ID NO: 324-383, respectively.)
- [32] Fig. 23 shows patient characteristics in one of the studies
- [33] Figs. 24A-24C compare CEA and ctDNA levels for different patients

DETAILED DESCRIPTION OF THE INVENTION

- [34] Colorectal cancer (CRC) is the second leading cause of cancer-related deaths in the United States. CRC can generally be cured by surgical excision if detected at any stage prior to distant metastasis to the liver and other organs. Unfortunately, about 35% of patients have such distant metastases, either occult or detectable, at the time of diagnosis, accounting for virtually all the deaths from the disease. The value of screening tests for colorectal neoplasia, particularly colonoscopy, has been highlighted in a variety of public awareness campaigns in the last several years. This has likely contributed to the decline

in CRC-related deaths, but the large number of individuals still being diagnosed with surgically incurable cancers attests to the fact that current efforts in this regard are inadequate. In particular, there is an urgent need for non-invasive tests that can complement colonoscopy and other invasive procedures and that can be offered to patients who are hesitant to undergo such inconvenient and invasive procedures. This need has stimulated the development of new tests for early detection, including virtual colonoscopy, improved assays for the presence of blood in stool, immunohistologic tests for cancer cells or proteins in stool, and DNA-based tests for genetic or epigenetic alterations (Ouyang DL, Chen JJ, Getzenberg RH, Schoen RE. Noninvasive testing for colorectal cancer: a review. *Am J Gastroenterol* 2005;100:1393-403.).

- [35] Mutant DNA molecules offer unique advantages over cancer-associated biomarkers because they are so specific. Though mutations occur in individual normal cells at a low rate ($\sim 10^{-9}$ to 10^{-10} mutations/bp/generation), such mutations represent such a tiny fraction of the total normal DNA that they are orders of magnitude below the detection limit of any test that has yet been described (including the one used in the current study). There is only one circumstance when a specific somatic mutation is present in an *appreciable* amount in any clinical sample: when it occurs in clonal fashion, *i.e.*, when the mutation is present in all cells of a specific population, thereby defining a neoplastic lesion.
- [36] Several studies have shown that mutant DNA can be detected in stool, urine, and blood of CRC patients (Osborn NK, Ahlquist DA. Stool screening for colorectal cancer: molecular approaches. *Gastroenterology* 2005;128:192-206). Moreover, technical factors that have limited the sensitivity of such assays are gradually being overcome. For example, improvements for stool-based testing include DNA stabilization after defecation (Olson J, Whitney DH, Durkee K, Shuber AP. DNA stabilization is critical for maximizing performance of fecal DNA-based colorectal cancer tests. *Diagn Mol Pathol* 2005;14:183-91.), removal of PCR inhibitors and bacterial DNA, cost-effective purification of sufficient amounts of human DNA for analysis (Whitney D, Skoletsky J, Moore K, Boynton K, Kann L, Brand R, Syngal S, Lawson M, Shuber A. Enhanced retrieval of DNA from human fecal samples results in improved performance of colorectal cancer

screening test. J Mol Diagn 2004;6:386-95) and the continuing delineation of mutant genes that can be assessed (Kann L, Han J, Ahlquist D, Levin T, Rex D, Whitney D, Markowitz S, Shuber A. Improved marker combination for detection of de novo genetic variation and aberrant DNA in colorectal neoplasia. Clin Chem 2006;52:2299-302.). Moreover, assays for detecting mutations have been developed that query each template molecule individually, dramatically increasing the signal to noise ratio. Such "digital" assays are particularly well-suited for the analysis of DNA in clinical samples such as stool or plasma because the mutant DNA fragments in such samples are greatly outnumbered by normal DNA fragments.

- [37] The inventors have developed methods for monitoring tumor burden in cancer patients. By detection of circulating tumor DNA in the patient, predictions regarding tumor recurrence can be made. Based on the predictions, treatment and surveillance decisions can be made. For example, circulating tumor DNA which indicates a future recurrence, can lead to additional or more aggressive therapies as well as additional or more sophisticated imaging and monitoring. Circulating DNA refers to DNA that is ectopic to a tumor.
- [38] Samples which can be monitored for "circulating" tumor DNA include blood and stool. Blood samples may be for example a fraction of blood, such as serum or plasma. Similarly stool can be fractionated to purify DNA from other components. Tumor samples are used to identify a somatically mutated gene in the tumor that can be used as a marker of tumor in other locations in the body. Thus, as an example, a particular somatic mutation in a tumor can be identified by any standard means known in the art. Typical means include direct sequencing of tumor DNA, using allele-specific probes, allele-specific amplification, primer extension, etc. Once the somatic mutation is identified, it can be used in other compartments of the body to distinguish tumor derived DNA from DNA derived from other cells of the body. Somatic mutations are confirmed by determining that they do not occur in normal tissues of the body of the same patient. Types of tumors which can be monitored in this fashion are virtually unlimited. Any tumor which sheds cells and/or DNA into the blood or stool or other bodily fluid can be

used. Such tumors include, in addition to colorectal tumors, tumors of the breast, lung, kidney, liver, pancreas, stomach, brain, head and neck, lymphatics, ovaries, uterus, bone, blood, etc.

- [39] Total DNA in a test sample can be determined by any means known in the art. There are many means for measuring total DNA. As detailed below, one method that can be used is a real-time PCR assay. Any gene or set of genes can be amplified. The LINE-1 gene family was employed because it is highly repeated and therefore requires a small sample to measure. The total DNA is measured so that measurements of tumor DNA collected at different times from a patient can be normalized. While genome equivalents can be used as a unit to express the total DNA content, other units of measurement can be used without limitation.
- [40] Because the amount of ectopic tumor DNA in a sample is very small, a highly sensitive means of measurement is desired. The measurement means described in detail below employs amplification on beads in an emulsion. The measurement means, called BEAMing, can detect mutations in stool and plasma DNA from patients with colorectal cancers (Fig. 5A-5B). BEAMing was named after its components - beads, emulsions, amplification, and magnetics – and essentially converts single DNA template molecules to single beads containing tens of thousands of exact copies of the template (Dressman D, Yan H, Traverso G, Kinzler KW, Vogelstein B. Transforming single DNA molecules into fluorescent magnetic particles for detection and enumeration of genetic variations. Proc. Natl. Acad. Sci. U S A 2003; 100:8817-22; U.S. Serial No. 10/562,840). This method permits one to determine how frequently mutations could be detected in the DNA from plasma or stool of the same patients as well as to investigate other parameters that could be useful in designing clinically applicable DNA-based tests in the future. Other measurement means can be used, if sufficiently sensitive. The mutant sequence which is first identified in the patient's tumor DNA is assayed in the ectopic body sample, such as blood (*e.g.*, serum or plasma), or stool. An easily collected sample is desirable. The ectopic body sample is one into which the particular type of tumor in the patient would

drain. Other body samples may include saliva, broncho-alveolar lavage, lymph, milk, tears, urine, cerebrospinal fluid, etc.

- [41] The sequence that is identified as somatically mutated in the tumor DNA of the patient is specifically determined in the ectopic body sample. Similarly, the corresponding sequence that is found in the patient's other body samples is also specifically determined. Thus, for example, if a tumor mutation at nucleotide X of gene *ABC* is a G nucleotide in the tumor and a T nucleotide in other body tissues, then both the G and the T versions of nucleotide X of gene *ABC* can be specifically measured and quantified in the ectopic body sample. One means of assessing these is with allele-specific hybridization probes. Other techniques which achieve sufficient sensitivity can be used.
- [42] Calculation of the number of mutant sequences (or the ratio of mutant to not-mutant sequences) can optionally be normalized to the total DNA content, *e.g.*, genome equivalents. The tumor burden index reflects the number of mutant (tumor) DNA molecules present in a test sample. The number of non-mutant DNA molecules in a sample may be included in the calculation of the tumor burden index to form a ratio. The normalization and/or ratio can be calculated by special purpose computer or general purpose computer or by human. The ratio can be recorded on paper, magnetic storage medium, or other data storage means. The normalized value is a data point to assess tumor burden in the whole individual. Additional assessments at different time points can optionally be made to obtain an indication of increase, decrease, or stability. The time points can be made in connection with surgery, chemotherapy, radiotherapy, or other form of therapy.
- [43] After tumor resection, if complete, a drastic decrease in tumor burden will be observed. However, if residual tumor remains, the tumor burden index will still be high or detectable. Because the half-life of ectopic DNA such as in the blood is fairly short, one can quickly assess surgical results using this technique. Incomplete resection can be detected in this means after 2 hours, 4 hours, 8 hours, 12 hours, 16 hours, 24 hours, 2 days, 3 days, 5 days, 7 days, 14 days, 21 days, 28 days, 56 days, etc. Incomplete tumor

resection may lead to increased monitoring, additional surgery, additional chemotherapy, additional radiation, or combinations of therapeutic modalities. Additional therapies may include increased dosage, frequency, or other measure of aggressiveness.

- [44] Genes in which mutations can be identified are any which are subject to somatic mutation in a patient's tumor. For ease of assay development, genes which are frequently subject to such mutations may be used. These include genes which are tumor suppressors or oncogenes, genes involved in cell cycle, and the like. Some commonly mutated genes in cancers which may be used are *APC*, *KRAS*, *TP53*, and *PIK3CA*. This list is not exclusive.
- [45] While any means of detection of mutations can be used, hybridization to allele-specific nucleic acid probes has been found to be effective. Prior to hybridization, double stranded hybridization reagents are typically heated to denature or separate the two strands, making them accessible to and available for hybridization to other partners. Slow cooling, *i.e.*, at least as slow as 1 degree C per second, at least as slow as 0.5 degree C per second, at least as slow as 0.25 degree C per second, at least as slow as 0.1 degree C per second, or at least as slow as 0.05 degree C per second, has been found useful. In addition, the presence of the reagent tetramethyl ammonium chloride (TMAC), has also been found to be useful, especially when one of the hybridization partners is attached to a bead.
- [46] Our results show that ctDNA is a promising biomarker for following the course of therapy in patients with metastatic colorectal cancer. ctDNA was detectable in all subjects before surgery, and serial blood sampling revealed oscillations in the level of ctDNA that correlated with the extent of surgical resection. Subjects who had detectable ctDNA after surgery generally relapsed within 1 year. The ctDNA seemed to be a much more reliable and sensitive indicator than the current standard biomarker (CEA) in this cohort of subjects.

- [47] Our studies are consistent with others that have shown that ctDNA can be detected in subjects with cancer, particularly in advanced tumors⁶. However, most such previous studies have not used techniques sufficiently sensitive to detect the low levels of ctDNA found in many of the subjects evaluated in the current study. Moreover, one of the crucial and distinguishing features of our approach lies in the ability to precisely measure the level of ctDNA rather than to simply determine whether or not ctDNA is detectable.
- [48] The results of our study suggest that ctDNA levels reflect the total systemic tumor burden, in that ctDNA levels decreased upon complete surgery and generally increased as new lesions became apparent upon radiological examination. However, whether ctDNA levels are exactly proportional to systemic tumor burden cannot be definitively determined, because there is no independent way to measure systemic total burden at this time. Radiographs are inaccurate, because lesions that are observed upon imaging are composed of live neoplastic cells, dead neoplastic cells and varying amounts of non-neoplastic cells (stromal fibroblasts, inflammatory cells, vasculature, and the like)¹¹. The proportion of these cell types in any lesion is unknown. Additionally, micrometastatic lesions that are smaller than a few millimeters, which in aggregate may make a large contribution to the total tumor burden, are not detectable by positron emission tomography, computed tomography or magnetic resonance imaging scans.
- [49] The approach used in our study can be considered a form of “personalized genomics.” As such, it has both advantages and disadvantages. The advantage over other biomarkers lies in its specificity, as the queried mutation should never be found in the circulation unless residual tumor cells are present somewhere in the subject's body. The disadvantage is that a marker specific for each subject must be developed. This entails the identification of mutations in the subject's tumor as a preliminary step (Figs. 1A-1B). Though we have performed this step with direct sequencing of DNA from paraffin-embedded tissues, it could be performed with simpler technologies, such as microarrays querying mutation hotspots^{12,13}. The second step—designing and testing a mutation-specific probe—is also time consuming at this stage of technological development. But it, too, could be simplified, in that a stock of probes, representing the most common mutations, could

WO 2010/014920

PCT/US2009/052436

easily be prepared in advance. This strategy may also be particularly useful for a different application of the approach, *i.e.*, cancer screening in a healthy population where mutational status is not known in advance.

[50] In sum, we present a framework for using circulating tumor DNA as a measure of tumor dynamics. The rationale is similar to that employed in the care of patients with HIV, in whom viral nucleic acids are quantitatively assessed to monitor asymptomatic disease and used to tailor therapy to the individual's needs. We envision that ctDNA could be used to noninvasively monitor many types of cancer and, as in the treatment of individuals with HIV, help influence clinical decision-making. As sequencing technologies improve, it will become relatively simple to identify such mutations in virtually any cancer. Indeed, such diagnostic applications are one of the major goals of the Cancer Genome Atlas project.

[51] The above disclosure generally describes the present invention.

A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only, and are not intended to limit the scope of the invention.

EXAMPLE 1

METHODS

[52] **Subjects and study design.** This study was approved by the Institutional Review Board of the Johns Hopkins Medical Institutions. Subjects were eligible if they had primary or metastatic colorectal cancer that was being treated surgically at The Johns Hopkins Sidney Kimmel Comprehensive Cancer Center. Between October 2005 and July 2006, 31 subjects diagnosed with colorectal cancer were screened during preoperative evaluation

for possible surgery. Twenty-eight subjects consented for the study, but seven of these were found not to be candidates for therapy, two subjects were lost during follow-up and one subject was found to have a medical condition other than colorectal cancer, leaving eighteen participants. Each subject agreed to have ctDNA assessed in plasma samples obtained before and after surgery and during prespecified intervals during their post-operative course (**Fig. 18**) through October 2007. We prospectively collected 162 plasma samples from the 18 subjects. Formalin-fixed paraffin-embedded tumor tissue was obtained from each subject and processed by the Surgical Pathology Laboratory at The Johns Hopkins Medical Institutes using routine procedures. We performed the analyses of the tumor tissues and the plasma samples in a blinded fashion once the clinical assessment was complete. We measured tumor sizes radiographically with computed tomography, and we used cross-sectional measurements in centimeters to estimate tumor burden.

- [53] **Isolation and quantification of DNA from plasma.** We drew peripheral blood into EDTA tubes (Becton Dickinson). Within one hour, we subjected the tubes to centrifugation at 820g for 10 min. We transferred 1-ml aliquots of the plasma to 1.5-ml tubes and centrifuged at 16,000g for 10 min to pellet any remaining cellular debris. We transferred the supernatant to fresh tubes and stored them at -80°C . We purified total genomic DNA from 2 ml of the plasma aliquots using the QIAamp MinElute virus vacuum kit (Qiagen) according to the manufacturer's instructions. We quantified the amount of total DNA isolated from plasma with a modified version of a human LINE-1 quantitative real-time PCR assay, as described previously¹⁴. Details are provided in
- [54] **Mutation analysis of DNA from tumor tissue.** We determined the mutation status of four genes in DNA purified from paraffin-embedded tumor tissue. We cut 10- μm sections and stained them with H&E. We used laser-capture microdissection to acquire neoplastic cells from these sections. We digested the dissected material overnight with proteinase K (Invitrogen) and purified genomic DNA from it with the QIAamp Micro Kit (Qiagen). We analyzed a total of 26 PCR products by direct sequencing. Further details concerning DNA amplification and sequencing are provided in Example 6.

- [55] **Mutation analysis of DNA from plasma.** We queried at least one mutation identified by sequencing of each subject's tumor tissue in plasma. In brief, we designed primers that could amplify the region containing the mutation for an initial amplification step with a high-fidelity DNA polymerase (New England BioLabs). We used the amplified product as a template in the subsequent BEAMing assay. The sequences of the primers and probes used for each test are listed in Example 6. The basic experimental features of BEAMing have been previously described¹⁵, and the modifications used in the current study are described in Example 6. We used the DNA purified from 2 ml plasma for each BEAMing assay. We repeated each measurement at least two times.
- [56] We used DNA purified from each subject's tumor as a positive control. We also included negative controls, performed with DNA from subjects without cancer, in each assay. Depending on the mutation being queried, the percentage of beads bound to mutant-specific probes in these negative control samples varied from 0.0061% to 0.00023%. This fraction represented sequence errors introduced by the high-fidelity DNA polymerase during the first PCR step, as explained in detail previously¹⁶. To be scored as positive in an experimental sample, the fraction of beads bound to mutant fragments had to be higher than the fraction found in the negative control, and the mean value of mutant DNA fragments per sample plus one standard deviation had to be >1.0. We analyzed bead populations generated by BEAMing at least twice for each plasma sample.
- [57] **Carcinoembryonic antigen measurement.** We analyzed CEA abundance by a two-step chemiluminescent microparticle immunoassay with the Abbott ARCHITECT i2000 instrument (Abbott Laboratories) at the Johns Hopkins Medical Institutions Clinical Chemistry Research Laboratory.
- [58] **Statistical analyses.** We quantified post-operative changes in ctDNA as a mean percentage decrease after surgery, with its standard error. We compared relative changes in CEA to ctDNA values with Student's unpaired *t*-test. We assessed changes from baseline with a one-sample *t*-test. The correlation between CEA and ctDNA levels was calculated with partial residuals from linear regression, taking into account within-patient

clustering. Recurrence was defined on the basis of radiographic and clinical findings. We calculated all confidence intervals at the 95% level. We performed computations were performed using JMP 6.0 software (SAS Institute) and SigmaPlot 10.0.1 (Systat Software).

EXAMPLE 2

Measurement of ctDNA

- [59] Quantification of circulating mutant ctDNA by BEAMing represents a personalized approach for assessing disease in subjects with cancer. The first step in this process is the identification of a somatic mutation in the subject's tumor (**Fig. 1A-1B**). Fig. 19 lists the characteristics of the subjects with colorectal cancer evaluated in this study. Four genes were assessed by direct sequencing in tumors from 18 subjects, and each of the tumors was found to have at least one mutation (**Fig. 20**).
- [60] The second step in the process is the estimation of the total number of DNA fragments in the plasma by real-time PCR (**Fig. 1A-1C**). Before surgery (day 0), there was a median of 4,000 fragments per milliliter of plasma in the 18 subjects described above (range between 10th and 90th percentiles, 1,810–12,639 DNA fragments ml^{-1}).
- [61] The third and final step is the determination of the fraction of DNA fragments of a given gene that contains the queried mutation. Such mutant DNA fragments are expected to represent only a small fraction of the total DNA fragments in the circulation. To achieve the sensitivity required for detection of such rare tumor-derived DNA fragments, we developed an improved version of BEAMing (detailed in Example 6). These improvements achieved high signal-to-noise ratios and permitted detection of many different mutations via simple hybridization probes under identical conditions. We attempted to design 28 assays, at least one for each of the 18 subjects, and were successful in every case. The median percentage of mutant DNA fragments in the 95 positive samples evaluated in this study was 0.18% (range between 10th and 90th

percentiles, 0.005–11.7%). Examples of typical assays from plasma serially collected from a representative subject are shown in **Figure 2A-2D**.

- [62] Multiplying the total number of DNA fragments of a gene in the analyzed volume of plasma (as determined by real-time PCR) by the fraction of mutant fragments (as determined by BEAMing) yields the number of mutant fragments (ctDNA number) in that volume of plasma (**Fig. 1C**). The median number of mutant DNA fragments in the 95 positive samples evaluated in this study was 39 (range between 10th and 90th percentiles, 1.3–1833.0).
- [63] The accuracy of these assays was assessed by measurements of the number of mutant DNA fragments derived from two different genes in the same subject. We were able to assay mutations in two different genes in 43 samples derived from nine study subjects. The ctDNA levels corresponding to the two mutant genes were found to be remarkably similar (correlation coefficient $R^2 = 0.95$, **Fig. 14**).

EXAMPLE 3

ctDNA dynamics in subjects with cancer undergoing therapy

- [64] We evaluated 18 subjects after a total of 22 surgeries during the course of this study (**Fig. 19**). The ctDNA level determined before surgery (day 0) varied widely, ranging from 1.3 to 23,000 mutant templates per sample (median 99 mutant templates per sample; range between 10th and 90th percentiles, 3–2,837). Seventeen of these surgeries involved complete resection of all evident tumor tissue, whereas five were incomplete resections. A sharp drop in the ctDNA level by the day of discharge (two to ten days after surgery) was observed in all subjects who underwent complete resections, with a 99.0% median decrease in ctDNA (range between 10th and 90th percentiles, 58.9–99.8%; **Fig. 24A-24C**). This decrease was already evident 24 h after surgery (96.7% median decrease, range between 10th and 90th percentiles, 31.4–100.0%). Through evaluation of a subject whose plasma

was sampled at multiple early times after complete resection, we estimated the half-life of ctDNA after surgery as 114 min (**Fig. 15**).

- [65] In the five cases with incomplete resections, the change in ctDNA was quite different. In two of these cases, the number of mutant fragments decreased only slightly at 24 h (55–56%), whereas in the other three cases, the number actually increased (141%, 329% and 794%). This increase was perhaps due to injury of remnant tumor tissue during the surgical procedure, with subsequent release of DNA. Surgically induced tissue injury is consistent with the observation that the total amount of DNA in the plasma (mutant plus normal) increased immediately after surgery in all subjects (**Fig. 16A-1 to 16E-3**).
- [66] Though the amount of ctDNA generally decreased after surgery, it did not decrease to undetectable levels in most cases. Plasma samples were available from the first follow-up visit, 13–56 d after surgery, in 20 instances. ctDNA was still detectable in 16 of these 20 instances, and recurrences occurred in all but one of these 16 (**Fig. 24A-24C**). In a marked contrast, no recurrence occurred in the four subjects in whom ctDNA was undetectable at the first follow-up visit. (**Fig. 24 A-24C**). The difference in recurrence rate between subjects with and without detectable ctDNA at the first follow-up was significant ($P = 0.006$ by Mantel-Cox log-rank test, **Fig. 3a**).
- [67] Representative time courses of ctDNA along with clinical and radiologic data on two subjects are provided in **Figure 4B-4C**, and similar data on all other subjects are shown in **Fig. 17**. Subjects 8 and 11 had more than one surgical procedure during the study, providing special opportunities to assess changes in ctDNA after repeated, controlled manipulation of tumor burden. Both of these subjects had incomplete resections in their initial surgery, and their ctDNA levels did not decrease (**Fig. 4B-4C**). They had complete resections in their second surgery, and the ctDNA abundance dropped precipitously thereafter. The ctDNA abundance then climbed back to higher levels over the next several months (**Fig. 4B-4C**).
- [68] Eleven of the subjects in our cohort received chemotherapy during the course of the study. In three of these subjects, ctDNA levels declined during the treatment. An

example is provided by subject 8: ctDNA decreased by more than 99.9%, whereas tumor volume (composed of live and dead neoplastic cells in addition to stromal cells) decreased only slightly (**Fig. 4A-4C**). In six subjects, there was an immediate rise in ctDNA after discontinuation of chemotherapy, as is evident in subjects 8 and 11 after the first chemotherapy (**Fig. 4A-4C**) and in subjects 1, 4, 10, and 12 (**Fig. 17**).

EXAMPLE 4

Comparison with carcinoembryonic antigen

[69] Carcinoembryonic antigen (CEA) is the standard biomarker for following disease in subjects with colorectal cancer and is routinely used in the management of the disease¹⁰. Only ten of the eighteen subjects had CEA levels $>5 \text{ ng ml}^{-1}$ (the boundary of the normal range) before study entry. (**Fig. 23**). This difference in sensitivity between the two assays (ctDNA versus CEA) was statistically significant; 56% versus 100%, respectively ($P = 0.008$, McNemar test). Moreover, even in those subjects with positive CEA levels before surgery, complete tumor resection resulted in a much less marked decrease in CEA than that observed with ctDNA (median decrease of 99.0% versus 32.5% in ctDNA versus CEA, respectively; $P < 0.001$, Student's *t*-test). There was a modest overall correlation between CEA abundance and ctDNA levels after correcting for clustering within subjects ($R^2 = 0.20$, $P < 0.001$, **Fig. 17**). Finally, when measured at the first post-operative follow-up visit on days 24–48, the ability of CEA levels to predict recurrent disease was less impressive than that of ctDNA levels ($P = 0.03$ by Mantel-Cox log-rank test, **Fig. 3b**).

EXAMPLE 5

Study design and collection of clinical samples

[70] For this study, specimens from subjects with colorectal cancer who had been acquired through a previous study were evaluated⁷. Subjects were at average risk for CRC as determined by family history and had no personal history of any type of cancer. Patients with non-specific abdominal symptoms or a history of basal cell or squamous cell carcinoma of the skin were not excluded. Stool and blood specimens were collected 6-12 days post-colonoscopy and prior to any bowel preparation for subsequent surgery. This study included 25 of the 40 previously identified cancer cases⁷ as 15 cases had inadequate amounts of residual material available. Patient characteristics are summarized in Table 1: Seven of the patients had stage I carcinomas, seven had stage II, eight had stage III, two had stage IV and one was of unspecified stage. The blood samples were drawn in BD Vacutainer tubes with EDTA (Becton Dickinson, Franklin Lakes, NJ USA) from 16 of the 25 patients. Plasma was prepared by centrifugation of blood at 1380 g for 30 min. The supernatant was transferred to a fresh tube and re-centrifuged. After centrifugation, the plasma was transferred to a Millipore Ultrafree-MC 0.45 micron filter device (Millipore, Billerica, MA, USA) to remove remaining cellular debris. The filter device was subjected to centrifugation at 1380 g for 15 min. The cleared plasma was transferred to a new tube and stored at -20° C until processed.

Identification of mutations in tumor tissue

[71] Tissues obtained upon surgical resection were used for mutation analysis, as reported previously^{5,4}. Briefly, snap-frozen or paraffin-embedded microdissected tumor tissue was used for the isolation of tumor DNA using the QIAamp DNA mini kit (Qiagen, Valencia, CA). All DNA *samples* were analyzed for 22 common mutations in *APC*, *TP53*, and *KRAS* using a single base extension (SBE) assay and a sequencing approach for exon 9 and 20 of *PIK3CA*, exon 3 of *CTNNB1*, and exon 15 of *APC*. The sequencing was performed by using single-stranded DNA templates in four separate sequencing reactions, each containing a R110 labeled AcyloTerminator nucleotide (PerkinElmer) and a mixture of ThermoSequenase (GE) and AcycloPol (PerkinElmer). Combined, the two marker panels were able to identify at least one mutation in the 24 tumor samples available for

this study (Table 2). The sensitivity of SBE and sequencing was 75% (18/24) and 79% (19/24), respectively (Fig. 13).

Isolation and quantification of stool DNA

- [72] Human DNA enriched for the target genes (*APC*, *TP53*, *KRAS*, and *PIK3CA*) was purified from total stool DNA using a Reversible Electrophoretic Capture Affinity Protocol (RECAP)⁸.
- [73] The copy number of gene fragments recovered from each stool sample was quantified using an iCycler™ IQ real-time PCR detection system (Biorad, Hercules, CA, USA). Duplicate reactions (50 µl) consisted of 5 µl of DNA, 10× PCR buffer (Takara Bio; Madison, WI, USA), 0.2 mM dNTPs (Promega, Madison, WI, USA), 0.5 µM of sequence-specific primers (sequences available upon request) and 2.5 U LATaq DNA polymerase (Takara Mirus Bio, Madison, WI, USA). The PCR parameters were 95°C for 3.5 min for denaturation followed by 40 cycles of 95° C for 1 min, 55° C for 1 min, and 72° C for 1 min.

DNA isolation and quantification of plasma DNA

- [74] DNA was purified from 2 ml plasma using the QIAamp MinElute Virus Vacuum Kit (Qiagen) as recommended by the manufacturer. The DNA was eluted in EB buffer (Qiagen), and stored at -20°C. The amount of total DNA isolated from plasma was quantified using a modified version of a human LINE-1 quantitative real-time PCR assay, as described previously⁹. Details are provided in Example 6.

Mutation analysis by BEAMing

- [75] Plasma and stool DNA was analyzed for somatic mutations by BEAMing. In total, 18 amplification primer sets were designed for the analysis of 33 different mutations. For each stool sample, a total of 30,000 genome equivalents were analyzed. One genome

equivalent was defined as 3.3 pg of genomic DNA and is equivalent to the DNA amount present in a haploid cell. A volume corresponding to the DNA purified from 2 ml of plasma was used for each BEAMing assay. The initial amplification was performed in multiples of 50 μ l PCR reactions, each containing template DNA equivalent to 250 μ l of plasma or 3,750 genome equivalents of stool DNA. Each reaction consisted of 5 \times Phusion high fidelity buffer, 1.5 U of Hotstart Phusion polymerase (both NEB), 0.2 μ M of each primer, 0.25 mM of each dNTP, and 0.5 mM MgCl₂. Nested PCR reactions were performed for selected target regions; for the second amplification, 2 μ l of the first PCR was added to a 20- μ l PCR reaction of the same makeup as described above except that different primers were used. Primer sequences and cycling conditions are listed in Fig. 9. PCR products were pooled, diluted, and quantified using the PicoGreen dsDNA assay (Invitrogen). The BEAMing procedure has been described previously¹⁰ and modifications used in the current study are described here.

- [76] A LSR II flow cytometry system (BD Bioscience) equipped with a high throughput autosampler was *used* for the analysis of each bead population. On average, 5×10^6 beads were analyzed for each plasma sample. The flow cytometric data was gated so that only single beads with extension products (as indicated by the control probe) were used for analysis. The mutation frequency was calculated as the number of gated beads attached to mutant sequences divided by the number of beads containing either mutant or wild-type sequences. In order for an assay to be scored as positive, it had to meet two criteria. First, the fraction of mutant beads had to be higher than the background emanating from polymerase errors arising during amplification. We used a Poisson distribution to estimate the expected variation in the background observed with DNA templates derived from normal lymphocyte DNA. A "positive" assay was scored as one in which the fraction was higher than 0.01 %. The second criterion was that the calculated number of mutant sequences in the templates used for analysis had to be ≥ 1 . For example, if in a sample, only 1,000 genomic equivalents were analyzed, yet the calculated fraction of beads bound to mutant sequences was 0.05 % (1 in 2,000), this

sample was scored as negative as the number of mutant template molecules was only 0.5 (0.05% x 1,000), which is less than 1.

Detection of Somatic Mutations by BEAMing

- [77] We assessed the performance of BEAMing for the detection of 33 different base changes in either *APC* (20), *KRAS* (4), *PIK3CA* (4), or *TP53* (5). The BEAMing procedure was performed as described previously with the important exception that an allele-specific hybridization (ASH) approach was developed for the analysis of bead-bound DNA (Fig. 1A-1C). The hybridization was performed with equimolar concentrations of fluorescently-labeled oligonucleotides complementary to the immobilized wild-type or mutant DNA sequences. Optimal allele discrimination for all 33 base changes was reached by an initial denaturation step followed by a slow cooling process in a tetramethylammonium chloride (TMAC) based buffer¹¹. All mutations we attempted to assess (transitions, transversions, insertions or deletions ranging from 1 to 5 bp) were successfully detected, with high signal to noise ratios, using this single TMAC-based hybridization procedure.
- [78] An example of ASH applied to beads generated by BEAMing is shown in Fig. 6. Positive control DNA populations were prepared using long oligonucleotides representing the genomic sequences, with the mutations in the center. Negative controls were prepared from DNA isolated from lymphocytes of healthy donors. Because no polymerase is completely error-free, mutations introduced during the initial amplification step create a small number of beads with mutant DNA sequences even when no mutant *DNA* templates are present in the sample DNA¹². In the current study, we used Phusion DNA polymerase (NEB) because it has been shown to have the lowest error rate of any commercially available enzyme tested¹². This background was individually determined for each mutation analyzed in the current study. The median background of mutations stemming from polymerase errors in normal lymphocyte DNA was 0.0009% (range 0.01% to 0.00013%). Variations in background rates were observed between and within genes, presumably reflecting the non-random nature of polymerase errors. Accordingly, an assay for a given sample was scored "positive for mutation" only if the mutant fraction was higher than the background by a conservative and statistically significant margin (see

Methods). Additionally, samples were scored as positive only if the calculated number of beads bound to mutant sequences was higher than a threshold defined by the genomic equivalents used in the assay, as also explained in more detail in the Methods.

Quantity and Quality of the DNA purified from stool

[79] Because BEAMing cannot only be used to detect mutant DNA templates but also to precisely quantify their abundance, it could be used to determine both the quantity and quality of cell-free *mutant* and normal DNA present in the stool of CRC patients. We therefore began the current study by analyzing the sizes of the mutant DNA fragments present in the stool of CRC patients. For this purpose, six PCR primer sets were designed for the amplification of DNA fragments that encompassed different *APC* mutations found in four patients with localized colorectal cancers. Two of the patients harbored Stage I and two harbored Stage II cancer (Fig. 11). The amplicon sizes obtained with these primers varied between 104 bp and 1,197 bp, with the mutations located in the middle of each amplicon (Fig. 7A). The DNA purified from an equal mass (181 mg) of stool was used in each assay. The number of mutant or normal template molecules was calculated by multiplying the respective fraction of beads bound to mutant or normal DNA sequences by the DNA concentrations measured by quantitative real-time PCR. In all four patients, the number of amplifiable normal DNA fragments decreased with increasing amplicon size. In patient 2, this decrease was only 3-fold whereas the decrease was more severe – up to a thousand-fold – in the other three patients (Fig. 7B). The mutant DNA fragments decreased with size in a similar, but not identical, fashion (Fig. 7C). As a result, the fraction of mutant DNA fragments was highest in the smallest amplicons (Fig. 7D); in patients 4 and 14, we could not detect *any* mutant DNA fragments when the largest amplicon size (1200 bp) was employed. These findings were important as they suggested that the sensitivity of tests for mutations in fecal DNA can be optimized by employing small amplicons. Based on this result, all the BEAMing assays used in the subsequent phases of this study were performed with ~100 bp amplicons whenever possible, and never longer than 126 bp (Fig. 9).

Mutation detection in stool DNA by BEAMing

[80] The clinicopathological characteristics of the 25 patients included in this study are summarized in Table 1.

Table 1. Patient characteristics

Characteristic	Value (n=25)
Age - yr	
Mean	67
Median (range)	66 (50-84)
Gender	
Female	15
Male	10
Stage	
I	7
II	7
III	8
IV	2
unknown	1
Differentiation	
Well	12
Moderate	9
Poor	3
Unspecified	1
Number of mutations in tumor tissue	
0	0
1	7
2	15
3	3

[81] Tumors ranged in size from 12-80 mm with a mean size of 41 mm (median 40 mm). Fourteen (56%) patients were early stage (Stage I or II), 10 (40%) were late stage (Stage III or IV), and one patient was of unknown stage. As outlined in the above, of the 24 patients where tumor tissue had been available, all had at least one mutation in the primary tumor (Fig. 13). For patient 25, where no tissue was available, two mutations were identified in stool DNA by the SBE assay (Fig. 13).

[82] Forty-five BEAMing assays were performed to assess the 33 different mutations in these samples (13 patients had at least one mutation found in another patient; Table 2). Of the 25 patients, 23 (92%, CI: 74%, 99%) had detectable levels of mutant DNA in their stool

samples. Mutations were detected as readily in patients with early stage colorectal cancers (Stages I and II) as in patients with late stage cancers (Stages III and IV) (Fig. 12). Interestingly, in one of the two patients in whom mutant DNA fragments could not be identified in the stool, the amount of normal DNA was very high (Patient 5).

- [83] The median fraction of mutant DNA present in stool samples was 0.32% but varied widely (range 0.0062% to 21.1%; Table 2).

Table 2:

Patient	Sex/ age, yr	Tumor			Mutation (codon)	Stool DNA			Plasma DNA			Score
		Stage (TNM)	Histology [†]	Site ²	Gene	Size, mm	Total DNA fragments per 362 mg Stool	Mut ant DNA , %	Sco re	Total DNA fragments per 2 ml Plasma	Mutant DNA , %	
1	M/66	I (T1N0M0)	Mod	R	APC	50	50,600	4.0	+			
2	F/64	I (T2N0M0)	Mod	Sig	APC	30	398,000	0.87	+	3676	0.000	-
3	F/70	I (T2N0M0)	Mod	C	TP53		302,000	0.20	+			
4	F/67	I (T1N0Mx)	Well	Tr	APC	45	1,808	0.71	+	2397	1.88	+
5	M/69	I (T2N0M0)	Well	Rs	APC	40	8,460	0.39	+	9317	0.013	+
					APC	24	1,030,000	0.00	-	3365	0.000	-
6	M/84	I (T2N0M0)	Mod	R	KRAS		1,030,000	0.00	-			
					KRAS			0				
7	F/58	I (T2N0Mx)	Well	Sig	APC	25	252,000	0.32	+			
					APC		252,000	0.78	+			
8	M/80	II (T3N0Mx)	Well	Sig	TP53	12	252,000	1.0	+			
					APC		13,840	1.0	+			
					PIK3CA		8,260	21	+			
					APC	25	7,420	0.00	-			
9	M/70	II (T3N0Mx)	Well	Sf	KRAS		7,420	0.21	+			
					APC	50	59,600	15.0	+	10652	0.002	-
10	F/58	II (T3N0M0)	Well	Tr	TP53	80	59,600	0.3	+			
11	F/65	II (T3N0M0)	Well	C	APC	50	113,800	1.17	+	4530	0.002	-
12	F/75	II (T3N0M0)	Well	R	APC	25	106,600	1.09	+			
13	M/80	II (T3N0Mx)	Well	As	APC	65	540,000	0.37	+	4650	0.42	+
14	M/66	II (T3N0M0)	Mod	R	APC	45	264,000	0.06	+			
					APC		264,000	0.04	+			
15	F/50	III (T4N1M0)	Mod	R	PIK3CA	25	15,740	0.2	+	3690	0.005	-
					APC		5,340	0.3	+			
16	M/64	III (T3N1M0)	Poor	Sig	KRAS	30	22,600	0.13	+	6422	0.062	+
17	M/74	III (T3N2M0)	Well	R	TP53	30	22,600	0.2	+	7047	0.033	+
					APC	30	7,140	0.05	+	2679	0.17	+
18	M/57	III (T3N1M0)	Mod/ Poor	Sig	KRAS		7,140	0.07	+			
19	F/65	III (T3N2Mx)	Mod/ Poor	As	PIK3CA	70	7,140	0.05	+			
20	M/59	III (T3N1Mx)	Well	Tr	APC	40	18,700	0.28	+			
21	M/73	III (T2N1Mx)	Mod	Tr	APC	42	17,460	1.3	+			
22	F/61	III (T3N1M0)	Mod	R	TP53	NR	5,920	0.18	+	11716	0.002	-
23	M/67	IV (T3N2M1)	Mod	Sig	KRAS	60	3,320	0.23	+			
24	M/65	IV (T3N1M1)	Well	As	TP53	30	3,320	0.10	+			
25	M/64	NR	NR	R	APC	35	11,320	0.00	+			
					KRAS		62	62				
					APC		11,320	0.3	+			
					APC		10,280	0.05	+	5043	0.007	-
					KRAS		7,200	0.23	+			
					APC		62,800	7.60	+	4206	0.001	-
					KRAS		62,800	4.43	+			
					PIK3CA		138,000	0.06	+	29233	6.6	+
					APC	30	254,000	0.62	+	4094	0.44	+
					PIK3CA		254,000	1.33	+			
					APC		356,000	0.90	+			
					TP53		356,000	10.2	+			

[illegible]

- [84] In most cases where two mutations could be assessed in the same stool sample, the fraction of mutant DNA molecules was similar. However, in four cases (patients 7, 8, 20 and 25) there was more than a 5-fold difference in the fraction of mutant DNA fragments from one gene compared to those in another gene.
- [85] Another important observation was that the median fraction of mutant DNA fragments in stool samples did not vary significantly across the stage of the patient's tumor: 0.83%, 0.31%, 0.20%, and 0.62% for Stage I, II, III, and IV, respectively (Fig. 8).
- [86] Finally, it was of interest to compare the results of BEAMing assays in these stool samples with those obtained previously using a modified sequencing approach⁵ and single base extension (SBE)⁷ (Fig. 13)). Of the 25 patients assessed in the current study, these assays combined were able to detect at least one mutation in only 15 patients (60% of the 25 analyzed) while BEAMing detected 23 (92% of the same 25 patients). This difference was statistically significant (Table 2, $p=0.008$, exact McNemar's test). The SBE assay alone, which comprises the component of a commercially available DNA test that assess 22 specific mutations in *APC*, *TP53*, and *KRAS*, performed about as well as the sequencing-based assay (60% (12/20) vs. 56% (10/18)). Our data also revealed a potential basis for the lower sensitivity of the SBE and sequencing tests compared to BEAMing. Those mutations that *were not* detected with these tests constituted 0.11% \pm 3.0% of the analyzed fragments. In contrast, those mutations that *were* detectable with SBE or sequencing were nine times more abundant (median 1.0% \pm 5.0%).

Mutation detection in stool and plasma DNA by BEAMing

- [87] Sixteen pairs of matched samples of blood and plasma were available for analysis. For each sample, one of the mutations found in the patients' tumor was selected for analysis. As noted in Table 2, 14 of these 16 (87.5%) patients' stool samples contained mutations at detectable levels. Mutant DNA fragments were found in a smaller proportion of the

plasma samples (8 of 16 [50%]; p-value for difference between the number of patients positive in *the* plasma and stool assays was 0.07 by the exact McNemar's test). There was only one patient that was negative for both tests (patient 5) and one patient with a negative stool test but a positive plasma test (patient 16). In patients that scored positive, the median fraction of mutant DNA was similar in stool (0.37%) and plasma (0.42%).

- [88] Though many previous studies have reported the presence of mutations in fecal DNA, this is the first to analyze them in a highly sensitive and quantitative manner. Similarly, other publications have reported the identification of genetic alterations in plasma or serum, but none have compared the results obtained with circulating DNA to those obtained with fecal DNA using identical techniques. The comparisons and quantifications reported here are important for guiding the development of sensitive and specific non-invasive screening tests for colorectal tumors in the future.
- [89] The quantitative analysis of fecal DNA highlighted several issues that are important for further research in this area. First, the highest sensitivities were realized when the amplicons were small, optimally less than 100 bp (Fig. 7A-7D). This is undoubtedly due to the DNA degradation that occurs either in cancer cells undergoing apoptosis or necrosis *in situ* or after they are released into the fecal stream. A similar size-dependence for DNA mutation detection has been described in plasma¹³. Note that this observation is not contradictory to studies showing that an increase in DNA integrity can be used as a marker for colorectal cancer¹⁴. Mutant DNA present in the stool of cancer patients represents only a minor fraction (median 0.32%; mean 1.89%) of the total DNA and therefore has little influence on the measurement of the integrity of the total (mutant plus normal) DNA. The observed increase of DNA integrity in cancer patients is most likely caused by the release of larger DNA fragments from normal cells within the tumor environment into the fecal stream. Indeed, recent results have shown that cancers are routinely infiltrated with particular types of inflammatory cells that could contribute relatively large DNA fragments of normal sequence¹⁵.

- [90] Second, the results make it clear that a minimum number of DNA template molecules must be obtained to realize the sensitivity afforded by BEAMing. The sensitivity of BEAMing for any of the analyzed mutations is such that at least one mutant template can be detected among 10,000 normal templates (0.01%). For some mutations, the sensitivity is as high as one mutant template among 800,000 normal templates (0.0013%). The sensitivity is only limited by the error rate of the polymerase used in the initial amplification¹². Utilization of this high technical sensitivity in practice, however, requires an adequate number of DNA templates. For example, if only 2,000 templates molecules are used per assay, then the maximum sensitivity that can be achieved is 0.05% rather than 0.01%. Obtaining this number of templates is not problematic with stool samples, but is often problematic for plasma. In the current study, 2 ml of plasma contained a median of 4,590 genome equivalents of DNA. This may be why the plasma-based assay was less sensitive (60%) than the stool-based assay (88%) in the same patients. To routinely obtain 30,000 genome equivalents from plasma (the number employed for the stool-based tests), 50 ml of blood would be necessary. Though this may be feasible in future prospective studies, it is unlikely to be available in retrospective studies such as ours.
- [91] Though stool provides a nearly limitless supply of DNA, there are other technical issues that affect the assay results. For example, stool contains a variety of PCR-inhibitors and a large excess of bacterial DNA, necessitating sequence-specific capture of human genomic DNA. Cost-effective methods for such capture have been developed and were used in the current study. However, they have not yet been optimized for the isolation of *small* DNA fragments that contain the mutations of interest. As shown in Fig. 7A-7D, the sizes of normal and mutant DNA fragments corresponding to specific genetic regions is not necessarily the same. The fraction of mutant fragments as a function of size is likely to vary both with the particular mutation in a patient-specific manner, as it depends both on the source of the normal DNA as well as the extent of degradation of the tumor DNA fragments. This issue could have affected our results in two ways. First, it could be responsible for the wide variations among the fractions of mutant fragments observed

within two different genes in some patients (*e.g.*, Patient 7 in Table 2). Second, it could explain why we were unable to detect mutations in some patients. For example, one of these two patients (Patient 5) had a very large number of normal fragments in his stool, more than two-fold that of any other patient. Optimization of the capture probes could in the future increase sensitivity over and above the 92% obtained in the current study.

[92] The new results also inform discussion of the relative advantages and disadvantages of stool *vs.* plasma analysis for early detection. As noted above, it is easier to obtain sufficient amounts of DNA from stool than from plasma. However, plasma is more convenient to collect from a practical standpoint, as it can be obtained during routine office visits, and it is easier to purify DNA from plasma than from stool. The sensitivity of detecting mutations in plasma from colorectal cancer patients (50%) is less than that in stool, but this could perhaps be increased by using more plasma in each assay. Perhaps the greatest advantage of stool versus plasma, however, is in the relative fractions of mutations observed in the feces of patients with different stage tumors. As shown in Figs. 8 and 12, the fraction of mutations in stool of early stage patients was as high as that in late stage patients. In contrast, our previous studies have shown that the fraction of mutations in the plasma of early stage patients is considerably lower than that of late stage patients (not apparent in the current study due to the small numbers of patients with positive plasma samples)¹³. Furthermore, the situation is likely to be even more pronounced in patients with large adenomas, as mutant DNA is much more difficult to detect in the plasma than in the stool of patients with these benign, but clinically significant, neoplasms.

[93] Though our study represents a step towards clinical implementation of a new, more sensitive and quantitative assay than currently available commercially, several additional steps will be necessary to realize this goal. In addition to clinical studies employing large numbers of patients with varying stage colorectal tumors and equally large numbers of controls, there are still technical issues to be overcome. In particular, cost-effective methods for querying a panel of genetic markers with BEAMing must be developed. In this regard, it is notable that mutations in all 25 patients in the current study were revealed

WO 2010/014920

PCT/US2009/052436

by the study of a relatively small number of common mutations. We envision that nearly 86% of patients with either colorectal cancer or large adenomas would harbor at least one of the 100 most common mutations. Implementation of such an assay would include parallel capture of ~10 exons and the subsequent multiplex PCR amplification of these DNA fragments. The newly described hybridization-based approach for mutation detection has also an advantage in that it can be easily automated. Next generation sequencing has the potential to further simplify the approach; the beads obtained by BEAMing can be analyzed by sequencing rather than by flow cytometry¹⁶. Additionally, the mutation marker panel could be reduced in size by including epigenetic markers¹⁷. Indeed, the lessons learned from the current study could be applied to optimize quantitative assays for methylation-based BEAMing or for any other tests for tumor-specific DNA variations that are developed in the future.

References for EXAMPLE 5

1. Ouyang DL, Chen JJ, Getzenberg RH, Schoen RE. Noninvasive testing for colorectal cancer: a review. *Am J Gastroenterol* 2005;100:1393-403.
2. Osborn NK, Ahlquist DA. Stool screening for colorectal cancer: molecular approaches. *Gastroenterology* 2005;128:192-206.
3. Olson J, Whitney DH, Durkee K, Shuber AP. DNA stabilization is critical for maximizing performance of fecal DNA-based colorectal cancer tests. *Diagn Mol Pathol* 2005;14:183-91.
4. Whitney D, Skoletsky J, Moore K, Boynton K, Kann L, Brand R, Syngal S, Lawson M, Shuber A. Enhanced retrieval of DNA from human fecal samples results in improved performance of colorectal cancer screening test. *J Mol Diagn* 2004; 6:386-95.
5. Kann L, Han J, Ahlquist D, Levin T, Rex D, Whitney D, Markowitz S, Shuber A. Improved marker combination for detection of de novo genetic variation and aberrant DNA in colorectal neoplasia. *Clin Chem* 2006; 52:2299-302.
6. Dressman D, Yan H, Traverso G, Kinzler KW, Vogelstein B. Transforming single DNA molecules into fluorescent magnetic particles for detection and enumeration of genetic variations. *Proc Natl Acad Sci U S A* 2003;100:8817-22.; U.S. Serial No. 10/562,840

7. Itzkowitz SH, Jandorf L, Brand R, Rabeneck L, Schroy PC, 3rd, Sontag S, Johnson D, Skoletsky J, Durkee K, Markowitz S, Shuber A. Improved fecal DNA test for colorectal cancer screening. *Clin Gastroenterol Hepatol* 2007;5:111-7.
8. Kent Moore J, Smith JA, Whitney DH, Durkee KH, Shuber AP. An electrophoretic capture method for efficient recovery of rare sequences from heterogeneous DNA. *Biotechniques* 2008;44:363-74.
9. Rago C, Huso DL, Diehl F, Karim B, Liu G, Papadopoulos N, Samuels Y, Velculescu VE, Vogelstein B, Kinzler KW, Diaz LA, Jr. Serial Assessment of Human Tumor Burdens in Mice by the Analysis of Circulating DNA. *Cancer Res* 2007;67:9364-9370.
10. Diehl F, Li M, He Y, Kinzler KW, Vogelstein B, Dressman D. BEAMing: single-molecule PCR on microparticles in water-in-oil emulsions. *Nat Methods* 2006;3:551-9.
11. Wood WI, Gitschier J, Lasky LA, Lawn RM. Base composition-independent hybridization in tetramethylammonium chloride: a method for oligonucleotide screening of highly complex gene libraries. *Proc Natl Acad Sci U S A* 1985;82:1585-8.
12. Li M, Diehl F, Dressman D, Vogelstein B, Kinzler KW. BEAMing up for detection and quantification of rare sequence variants. *Nat Methods* 2006;3:95-7.
13. Diehl F, Li M, Dressman D, He Y, Shen D, Szabo S, Diaz LA, Jr., Goodman SN, David KA, Juhl H, Kinzler KW, Vogelstein B. Detection and quantification of mutations in the plasma of patients with colorectal tumors. *Proc Natl Acad Sci U S A* 2005;102:16368-16373.
14. Boynton KA, Summerhayes IC, Ahlquist DA, Shuber AP. DNA integrity as a potential marker for stool-based detection of colorectal cancer. *Clin Chem* 2003;49:1058-65.
15. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, Tosolini M, Camus M, Berger A, Wind P, Zinzindohoue F, Bruneval P, Cugnenc PH, Trajanoski Z, Fridman WH, Pages F. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 2006;313:1960-4.
16. Shendure J, Porreca GJ, Reppas NB, Lin X, McCutcheon JP, Rosenbaum AM, Wang MD, Zhang K, Mitra RD, Church GM. Accurate multiplex polony sequencing of an evolved bacterial genome. *Science* 2005;309:1728-32.

17. Chen WD, Han ZJ, Skoletskey J, Olson J, Sah J, Myeroff L, Platzner P, Lu S, Dawson D, Willis J, Pretlow TP, Lutterbaugh J, Kasturi L, Willson JK, Rao JS, Shuber A, Markowitz SD. Detection in fecal DNA of colon cancer-specific methylation of the nonexpressed vimentin gene. J Natl Cancer Inst 2005;97:1124-32.

EXAMPLE 6

Isolation of DNA from formalin-fixed, paraffin embedded (FFPE) tumor tissue

- [94] Eighteen tumor specimens were collected after liver or colon surgery, fixed in formalin, and embedded in paraffin. Ten μm sections were cut and mounted on PEN-membrane slides (Palm GmbH, Bernried, Germany). The sections were deparaffinized and stained with hematoxylin and eosin. All specimens underwent histological examination to confirm the presence of tumor tissue, which was dissected from completely dried sections with a MicroBeam laser microdissection instrument (Palm). The dissected tumor tissue was digested overnight at 60°C in 15 μl ATL buffer (Qiagen) and 10 μl Proteinase K (20 mg/ml; Invitrogen). DNA was isolated using the QIAamp DNA Micro Kit (Qiagen) following the manufacturer's protocol. The isolated DNA was quantified by hLINE-1 quantitative PCR as described below.

PCR amplification and direct sequencing of DNA isolated from tumor tissue

- [95] All DNA samples isolated from tumor tissue were analyzed for mutations in 26 regions of *APC* (19), one region of *KRAS* (1), two regions of *PIK3CA* (2), and four regions of *TP53* (4) using direct Sanger sequencing. Due to degradation of DNA in FFPE tissue, the amplicon sizes were chosen to be between 74 to 132 bp in length. The first PCR was performed in a 10 μl reaction volume containing 50-100 genome equivalents (GEs) of template DNA (1 GE equals 3.3 pg of human genomic DNA), 0.5 U of Platinum Taq DNA Polymerase (Invitrogen), 1 \times PCR buffer (67 mM of Tris-HCl, pH 8.8, 67 mM of MgCl_2 , 16.6 mM of $(\text{NH}_4)_2\text{SO}_4$, and 10 mM of 2-mercaptoethanol), 2 mM ATP, 6% (v/v) DMSO, 1 mM of each dNTP, and 0.2 μM of each primer. The sequences of the primer sets are listed in Fig. 20. The amplification was carried out under the following conditions: 94°C for 2 min; 3 cycles of 94°C for 15 s, 68°C for 30 s, 70°C for 15 s; 3 cycles of 94°C for 15 s, 65°C for 30 s, 70°C for 15 s, 3 cycles of 94°C for 15 s, 62°C for 30 s, 70°C for 15 s; 40 cycles of 94°C for 15 s, 59°C for 30 s, 70°C for 15 s. One

microliter of the first amplification was then added to a second 10- μ l PCR reaction mixture of the same makeup as the one described above, except that different primers were used (Fig. 10). The second (nested) PCR reaction was temperature cycled using the following conditions: 2 min at 94°C; 15 cycles of 94°C for 15 s, 58°C for 30 s, 70°C for 15 s. The PCR products were purified using the AMPure system (Agencourt, Beverly, MA) and sequenced from both directions using BigDye Terminator v3.1 (Applied Biosystems). The primers used for sequencing had a 30 bp polyT tag attached to the 5' prime end to improve the sequence quality for the first 30 bases (Tag1 primer: 5'-(dT)₃₀-tccgcgaaattaatacgac; SEQ ID NO: 1; M13 primer: 5'-(dT)₃₀-gtaaaacgacggccagt; SEQ ID NO: 3). Sequencing reactions were resolved on an automated 96-capillary DNA sequencer (Spectrumedix, State College, PA). Data analysis was performed using Mutation Explorer (SoftGenetics, State College, PA).

Quantification of total plasma DNA by quantitative real-time PCR

[96] The amount of total DNA isolated from plasma samples was quantified using a modified version of a human LINE-1 quantitative real-time PCR assay¹. Three primer sets were designed to amplify differently sized regions within the most abundant consensus region of the human LINE-1 family (79 bp for: 5'-agggacatggatgaaattgg; SEQ ID NO: 4. 79bp rev: 5'-tgagaatatgcggtgtttgg; SEQ ID NO: 5; 97 bp for: 5'-tggcacatataccatggaa; SEQ ID NO: 6, 97 bp rev: 5'-tgagaatgatggtttccaatttc; SEQ ID NO: 7; 127 bp for: 5'-acttggaaaccaacccaaatg; SEQ ID NO: 8, 127 bp rev: 5'-tcatccatgtccctacaaagg; SEQ ID NO: 9). PCR was performed in a 25 μ l reaction volume consisting of template DNA equal to 2 μ l of plasma, 0.5 U of Platinum Taq DNA Polymerase, 1 \times PCR buffer (see above), 6% (v/v) DMSO, 1 mM of each dNTP, 1:100,000 dilution of SYBR Green I (Invitrogen), and 0.2 μ M of each primer. Amplification was carried out in an iCycler (Bio-Rad) using the following cycling conditions: 94°C for 1 min; 2 cycles of 94°C for 10 s, 67°C for 15 s, 70° C for 15 s; 2 cycles of 94° C for 10 s, 64° C for 15 s, 70° C for 15 s, 2 cycles of 94° C for 10 s, 61° C for 15 s, 70° C for 15 s; 35 cycles of 94° C for 10 s, 59° C for 15 s, 70° C for 15 s. Various dilutions of normal human lymphocyte DNA were incorporated in each plate setup to serve as standards. The threshold cycle number was determined using

Bio-Rad analysis software with the PCR baseline subtracted. Each quantification was done in duplicate. The total DNA was calculated using the LINE-1 amplicon closest in size to the amplicon being evaluated for mutations (Fig. 11). When the amplicon was equally close to two different LINE-1 amplicons, the average concentration was used. In control experiments with plasma, we found that the number of genome equivalents assessed by the assay of LINE sequences was highly correlated with the number of genome equivalents (GE) of *APC*, *KRAS*, *PIK3CA*, or *RAS*. LINE sequence-based assays, rather than these individual genes, were chosen to measure GE because it required a much smaller amount of plasma to measure GE with the former because of its highly repeated nature in the genome.

BEAMing

- [97] Twelve different primer sets were designed for the analysis of 20 mutations (Fig. 11). The DNA purified from 2 ml of plasma was used for each BEAMing assay. An initial amplification with a high fidelity DNA polymerase was performed in eight separate 50 μ l PCR reactions each containing template DNA from 250 μ l of plasma, 5 \times Phusion High Fidelity PCR buffer (NEB), 1.5 U of Hotstart Phusion polymerase (NEB), 0.2 μ M of each primer, 0.25 mM of each dNTP, and 0.5 mM MgCl₂. Temperature cycling was carried out as described in Fig. 11. Using the primers listed in Fig. 11, a second PCR (nested) was performed by adding 2 μ l of the first amplification to a 20- μ l PCR reaction of the same makeup as the first one. PCR products were pooled, diluted, and quantified using the PicoGreen dsDNA assay (Invitrogen). The fluorescence intensity was measured using a CytoFluor multiwell plate reader (PE Biosystems) and the DNA quantity was calculated using Lambdaphage DNA reference standards.
- [98] Emulsion PCR was performed as described previously². Briefly, a 150 μ l PCR mixture was prepared containing 18 pg template DNA, 40 U of Platinum Taq DNA polymerase (Invitrogen), 1 \times PCR buffer (see above), 0.2 mM dNTPs, 5 mM MgCl₂, 0.05 μ M Tag1 (5'-tcccgcgaaattaatacgac; SEQ ID NO: 1), 8 μ M Tag2 (5'-gctggagctctgcagcta; SEQ ID

NO: 2) and $\sim 6 \times 10^7$ magnetic streptavidin beads (MyOne, Invitrogen) coated with Tag1 oligonucleotide (5' dual biotin-T-Spacer18- tcccgcgaaattaatacgac; SEQ ID NO: 1). The 150 μ l PCR reaction, 600 μ l oil/emulsifier mix (7% ABIL WE09, 20% mineral oil, 73% Tegosoft DEC, Degussa Goldschmidt Chemical, Hopewell, VA), and one 5 mm steel bead (Qiagen) were added to a 96 deep well plate 1.2 ml (Abgene). Emulsions were prepared by shaking the plate in a TissueLyser (Qiagen) for 10 s at 15 Hz and then 7 s at 17 Hz. Emulsions were dispensed into eight PCR wells and temperature cycled at 94°C for 2 min; 3 cycles of 94°C for 10 s, 68°C for 45 s, 70°C for 75 s; 3 cycles of 94°C for 10 s, 65°C for 45 s, 70°C for 75 s, 3 cycles of 94°C for 10 s, 62°C for 45 s, 70°C for 75 s; 50 cycles of 94°C for 10 s, 59°C for 45 s, 70°C for 75 s.

- [99] To break the emulsions, 150 μ l breaking buffer (10 mM Tris-HCl, pH 7.5, 1% Triton-X 100, 1% SDS, 100 mM NaCl, 1 mM EDTA) was added to each well and mixed with a TissueLyser at 20 Hz for 20 s. Beads were recovered by spinning the suspension at 3,200 g for 2 min and removing the oil phase. The breaking step was repeated twice. All beads from 8 wells were consolidated and washed with 150 μ l wash buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl). The DNA on the beads was denatured for 5 min with 0.1 M NaOH. Finally, beads were washed with 150 μ l wash buffer and resuspended in 150 μ l of the same buffer.
- [100] The mutation status of DNA bound to beads was determined by allele-specific hybridization. Fluorescently labeled probes complementary to the mutant and wild-type DNA sequences were designed for 20 different mutations. The size of the probes ranged from 15 bp to 18 bp depending on the GC content of the target region. All mutant probes were synthesized with a Cy5TM fluorophore on the 5' end and all wild-type probes were coupled to a Cy3TM fluorophore (Integrated DNA Technologies, Coralville, IA, or Biomers, Ulm, Germany). In addition, oligonucleotides that bound to a separate location within the amplicon were used to label every extended PCR product as a positive control. These amplicon specific probes were synthesized with a ROXTM fluorophore attached to their 5' ends. Probe sequences are listed in Fig. 12. Each allele-specific hybridization reaction contained $\sim 1 \times 10^7$ beads present in 30 μ l wash buffer (see above), 66 μ l of $1.5 \times$

WO 2010/014920

PCT/US2009/052436

hybridization buffer (4.5 M tetramethylammonium chloride, 75mM Tris-HCl pH 7.5, 6 mM EDTA), and 4 µl of a mixture of mutant, wild-type, and gene-specific fluorescent probes, each at 5 µM in TE buffer. The hybridization mixture was heated to 70°C for 10 s and slowly (0.1°C/s) cooled to 35°C. After incubating at 35°C for 2 min, the mixture was cooled (0.1°C/sec) to room temperature. The beads were collected with a magnet and the supernatant containing the unbound probes was removed using a pipette. The beads were resuspended in 100 µl of 1× hybridization buffer and heated to 48°C for 5 min to remove unbound probes. After the heating step, beads were again separated magnetically and washed once with 100 µl wash buffer. In the final step, the supernatant was removed and beads resuspended in 200 µl TE buffer for flow cytometric analysis.

- [101] A LSR II flow cytometry system (BD Bioscience) equipped with a high throughput autosampler was used for the analysis of each bead population. An average of 5×10^6 beads were analyzed for each plasma sample. Beads with no extension product were excluded from the analysis. Negative controls, performed using DNA from patients without cancer, were included in each assay. Depending on the mutation being queried, the fraction of beads bound to mutant-specific probes in these negative control samples varied from 0.0061% to 0.00023%. This fraction represented sequence errors introduced by the high fidelity DNA polymerase during the first PCR step, as explained in detail previously³. To be scored as positive in an experimental sample, (i) the fraction of beads bound to mutant fragments had to be higher than the fraction found in the negative control, and (ii) the mean value of mutant DNA fragments per sample plus one standard deviation had to be >1.0. Bead populations generated by BEAMing were analyzed at least twice for each plasma sample.

References for example 6 only

1. Rago, C. et al. Serial Assessment of Human Tumor Burdens in Mice by the Analysis of Circulating DNA. *Cancer Res* 67, 9364-9370 (2007).
2. Diehl, F. et al. BEAMing: single-molecule PCR on microparticles in water-in-oil emulsions. *Nat Methods* 3, 551-9 (2006).
3. Li, M., Diehl, F., Dressman, D., Vogelstein, B. & Kinzler, K.W. BEAMing up for detection and quantification of rare sequence variants. *Nat Methods* 3, 95-7 (2006).

Individual Patient Summaries

Also see Fig.16A-1 to 16E-3

- [102] **Patient 1** originally underwent a low anterior resection for rectal carcinoma and was found to have multiple liver metastases with PET/CT scanning. They received post-operative with 5-fluorouracil, oxaliplatin (FOLFOX) and bevacizumab (**Chemotherapy**) for two cycles and repeat imaging revealed a good response. At the time of study entry, the patient underwent right hepatectomy and left lobe wedge resection and cholecystectomy (**Surgery**), followed by chemotherapy with 5-fluorouracil, leucovorin, oxaliplatin and bevacizumab (**Chemotherapy**). Repeat imaging revealed multiple new lung lesions and two new liver lesions. Various other chemotherapy regimens were utilized with continued progression of disease. The patient is currently being considered for a Phase I trial.
- [103] **Patient 2** was originally diagnosed with a T3N0M0 colon adenocarcinoma and underwent a left hemicolectomy. At the time of study entry, the patient underwent a right hepatic lobectomy and partial diaphragm resection for metastatic disease (**Surgery**). Repeat imaging studies revealed progressive disease three months following his liver lobectomy and the patient died of disease shortly thereafter.
- [104] **Patient 3** was initially found to have metastatic mucinous colon adenocarcinoma, T2N1M1 with a single liver metastasis who underwent a right hemicolectomy with planned liver resection (**Surgery**). However, the patient was found to have diffuse peritoneal implants at the time of surgery, and the liver resection was not performed. Post-operative CT scans revealed evidence of progressive disease with enlarging liver lesion and a new pulmonary nodule. The patient opted to proceed with supportive care only and died of disease approximately one year following his surgery.

- [105] **Patient 4** was diagnosed with metastatic colon adenocarcinoma. At study entry, 12 months following the initial surgery, the patient received pre-operative chemotherapy with 5- fluorouracil, oxaliplatin and bevacizumab. The patient then underwent a partial hepatectomy of two liver lesions with radio-frequency ablation of the margins with pathology concurrent with recurrent metastatic adenocarcinoma (**Surgery**). Subsequent CT scans have revealed no evidence of disease recurrence to date.
- [106] **Patient 6** originally presented with a T3N1M1 colon adenocarcinoma, and at the time of study entry underwent a right hepatectomy and right lower lobe lung wedge resection (**Surgery**). Follow-up CT scans revealed no evidence of disease and the patient was started on chemotherapy. Eight months later, repeat imaging then revealed a new liver metastasis. The patient then switched to irinotecan, 5-fluorouracil and bevacizumab (**Chemotherapy 1**), but despite four months of therapy still had persistent disease on follow-up CT scans. They were subsequently started on 5-fluorouracil, leucovorin, oxaliplatin and bevacizumab (**Chemotherapy 2**)
- [107] **Patient 7** has a prior history of a resected T3N2M1 rectosigmoid adenocarcinoma. At the time of study entry, the patient underwent surgical excision of two recurrent liver lesions, and an additional 4 liver lesions were treated with radiofrequency ablation (**Surgery**). Post-operative imaging revealed no evidence of disease, however, imaging three months later revealed new liver disease and new lung metastases. The patient was started on irinotecan, cetuximab, and bevacizumab (**Chemotherapy**). Despite chemotherapy, on follow-up imaging the patient was noted to have persistent and progressing disease.
- [108] **Patient 9** originally presented with a T3N1M0 colon adenocarcinoma followed by adjuvant 5-fluorouracil and leucovorin. At the time of study entry, a solitary liver lesion was noted, and the patient underwent a right hepatectomy, with pathology revealing recurrent adenocarcinoma (**Surgery**). The patient was given post-operative 5-

fluorouracil, oxaliplatin and bevacizumab (**Chemotherapy**) and follow up imaging has revealed no evidence of disease recurrence, with evidence of a fully regenerated liver.

- [109] **Patient 10** was originally diagnosed with metastatic colorectal adenocarcinoma to the liver and was treated with 5-fluorouracil, oxaliplatin and bevacizumab for four months (**Chemotherapy**). A right hepatectomy and right hemicolectomy was performed (**Surgery 1**). The liver resection was margin positive. Post-operative imaging revealed no evidence of disease. Repeat imaging performed three months later revealed 3 new left liver lesions and the patient subsequently underwent a left liver hepatectomy with radio-frequency ablation to the margins (**Surgery 2**). Post-operative imaging revealed no evidence of disease. At two months follow-up she was found to have boney metastases with a T7 compression fracture for which she underwent external beam radiation.
- [110] **Patient 12** was initially diagnosed with metastatic colon adenocarcinoma. At the time of study entry, the patient underwent a repeat partial hepatectomy with radio-frequency ablation (**Surgery**) after achieving some stabilization of disease with 5-fluorouracil, leucovorin, and oxaliplatin (**Chemotherapy**). Post-operative scans revealed no evidence of disease in the liver. However, CT scan of the chest revealed numerous new pulmonary lesions and a follow up PET showed new liver lesions as well. The patient was then referred for a Phase I clinical study.
- [111] **Patient 13** had a history of metastatic colon cancer resected from the sigmoid colon, liver and xiphoid process. Approximately 14 months after their original diagnosis, a CT scan revealed a 1cm lesion in the liver, and a follow-up PET scan showed two adjacent foci of disease near the left hepatic lobe. A CT scans performed three months later showed increase in size of the hepatic lesions and a new peritoneal implant. They then underwent resection of the recurrent disease with partial hepatectomy, partial gastrectomy, and partial omentectomy (**Surgery**). Follow-up CT scans performed 1-year following surgery showed hepatic and omental recurrences.

- [112] **Patient 14** was found to have colon adenocarcinoma on screening colonoscopy with CT scans showing no evidence of distant metastases. They underwent a sigmoid colectomy (**Surgery**) and pathology revealed a T3N0M0 tumor. No adjuvant chemotherapy was given and they were followed with serial CT scans. The last CT scan showed no evidence of disease.
- [113] **Patient 15** had a history of a completely resected T3N1Mx cecal mass and resected umbilical recurrence. Three years after the resection of the primary tumor, a CT scan of the abdomen then revealed a solitary liver metastasis. The patient underwent a right liver hepatectomy (**Surgery**). A follow-up CT scans one month later showed no evidence of disease, but the patient died of disease approximately one year later from recurrent metastatic disease.
- [114] **Patient 16** had a rectosigmoid mass on CT after being worked up for bright red blood per rectum, and underwent a sigmoid colectomy (**Surgery**). She was started on 5-fluorouracil, leucovorin and oxaliplatin, which she continued for the next five months (**Chemotherapy**). Follow-up CT scans following completion of therapy has shown no evidence of disease recurrence.
- [115] **Patient 17** is a patient with a history of resected colorectal cancer that was found by PET CT to have an isolated liver metastasis in the right lobe. They underwent a right hepatectomy (**Surgery**) and received post-operative chemotherapy with 5-fluorouracil, leucovorin, oxaliplatin and avastin (**Chemotherapy**). She was found to have a recurrence 7 months after surgery.
- [116] **Patient 18** was found to have a T3N1Mx adenocarcinoma after undergoing a low anterior resection for a rectal mass. Three years later the patient was noted to have a left hepatic

lobe lesion discovered on CT scan imaging. The patient underwent a laparoscopic liver resection (**Surgery**). He received no additional chemotherapy and is currently disease-free.

WO 2010/014920

PCT/US2009/052436

References for application excluding examples 5 and 6

1. Wood, L.D. *et al.* The genomic landscapes of human breast and colorectal cancers. *Science* **318**, 1108–1113 (2007).
2. Nagrath, S. *et al.* Isolation of rare circulating tumour cells in cancer patients by microchip technology. *Nature* **450**, 1235–1239 (2007).
3. Cristofanilli, M. *et al.* Circulating tumor cells, disease progression and survival in metastatic breast cancer. *N. Engl. J. Med.* **351**, 781–791 (2004).
4. Sidransky, D. Emerging molecular markers of cancer. *Nat. Rev. Cancer* **2**, 210–219 (2002).
5. Goebel, G., Zitt, M., Zitt, M. & Muller, H.M. Circulating nucleic acids in plasma or serum (CNAPS) as prognostic and predictive markers in patients with solid neoplasias. *Dis. Markers* **21**, 105–120 (2005).
6. Fleischhacker, M. & Schmidt, B. Circulating nucleic acids (CNAs) and cancer—a survey. *Biochim. Biophys. Acta* **1775**, 181–232 (2007).
7. Gormally, E., Caboux, E., Vineis, P. & Hainaut, P. Circulating free DNA in plasma or serum as biomarker of carcinogenesis: practical aspects and biological significance. *Mutat. Res.* **635**, 105–117 (2007).
8. Diehl, F. *et al.* Detection and quantification of mutations in the plasma of patients with colorectal tumors. *Proc. Natl. Acad. Sci. USA* **102**, 16368–16373 (2005).
9. Dressman, D., Yan, H., Traverso, G., Kinzler, K.W. & Vogelstein, B. Transforming single DNA molecules into fluorescent magnetic particles for detection and enumeration of genetic variations. *Proc. Natl. Acad. Sci. USA* **100**, 8817–8822 (2003).
10. Goldstein, M.J. & Mitchell, E.P. Carcinoembryonic antigen in the staging and follow-up of patients with colorectal cancer. *Cancer Invest.* **23**, 338–351 (2005).

11. Li, H., Fan, X. & Houghton, J. Tumor microenvironment: the role of the tumor stroma in cancer. *J. Cell. Biochem.* **101**, 805–815 (2007).
12. Hacia, J.G. & Collins, F.S. Mutational analysis using oligonucleotide microarrays. *J. Med. Genet.* **36**, 730–736 (1999).
13. Shendure, J., Mitra, R.D., Varma, C. & Church, G.M. Advanced sequencing technologies: methods and goals. *Nat. Rev. Genet.* **5**, 335–344 (2004).
14. Rago, C. *et al.* Serial Assessment of human tumor burdens in mice by the analysis of circulating DNA. *Cancer Res.* **67**, 9364–9370 (2007).
15. Diehl, F. *et al.* BEAMing: single-molecule PCR on microparticles in water-in-oil emulsions. *Nat. Methods* **3**, 551–559 (2006).
16. Li, M., Diehl, F., Dressman, D., Vogelstein, B. & Kinzler, K.W. BEAMing up for detection and quantification of rare sequence variants. *Nat. Methods* **3**, 95–97 (2006)
17. WO 05/10145 A3

WE CLAIM:

1. A method to monitor tumor burden over time, comprising the steps of:
measuring in a test sample of blood or stool of a cancer patient number of copies of DNA fragments of a gene that have a mutation, wherein the mutation is present in tumor tissue of the cancer patient but not in normal tissue of the patient, wherein the step of measuring is performed at a plurality of time points to monitor increase, decrease, or stability of the number of copies, and wherein a change in the number of copies is an index of a change in the tumor burden over time in the patient.
2. The method of claim 1 further comprising the step of: detecting the mutation in the gene in the tumor tissue.
3. The method of claim 2 further comprising the step of: testing normal tissue of the patient to determine the absence of the mutation in the gene in the normal tissue.
4. The method of claim 1 wherein the gene is frequently mutated in tumors but not in normal tissue of humans.
5. The method of claim 1 further comprising the steps of:
measuring number of copies of DNA fragments of the gene that do not have the mutation in the test sample; and
dividing the number of copies of DNA fragments that have the mutation by the number of copies of DNA fragments of the gene that do not have the mutation in the test sample, to provide a ratio.
6. The method of claim 5 further comprising the step of:
measuring total amount of DNA in the test sample of the cancer patient and normalizing the ratio to the total amount of DNA.
7. The method of claim 1 further comprising the step of:
recommending adjuvant therapy if DNA fragments of a gene that have the mutation are detected within 2 months of tumor resection.
8. The method of claim 1 further comprising the step of:

recommending adjuvant therapy if DNA fragments of a gene that have the mutation are detected within 1 week of tumor resection.

9. The method of claim 1 further comprising the step of:
recommending adjuvant therapy if DNA fragments of a gene that have a mutation are detected within 1 day of tumor resection.

10. The method of claim 1 further comprising the step of:
recommending adjuvant therapy if DNA fragments of a gene that have a mutation are detected after 2 days post tumor resection.

11. The method of claim 1 further comprising the step of:
predicting tumor recurrence if DNA fragments of a gene that have a mutation are detected after 2 days post tumor resection.

12. The method of claim 1 further comprising the step of:
recommending adjuvant therapy if DNA fragments of a gene that have a mutation are detected more than 4 hours after of tumor resection.

13. The method of claim 2 wherein in the step of detecting, the mutation is detected by nucleotide sequencing of tumor DNA of the cancer patient.

14. The method of claim 2 wherein in the step of detecting, the mutation is detected by hybridization to mutation specific nucleic acid probes.

15. The method of claim 1 wherein the mutation is in a gene that is *APC*, *KRAS*, *TP53*, or *PIK3CA*.

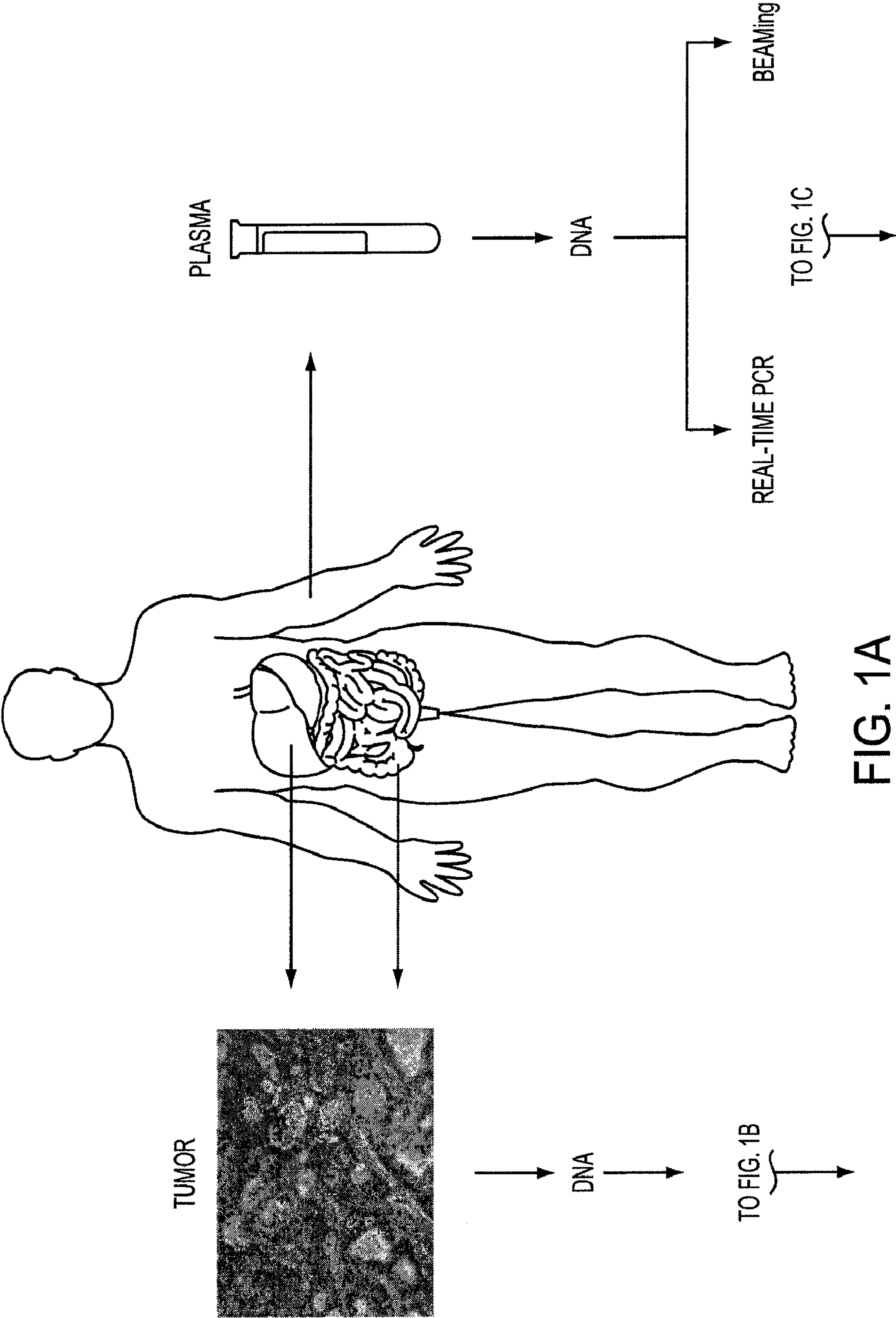
16. The method of claim 1 wherein the mutation is in a tumor suppressor gene or an oncogene.

17. The method of claim 1 wherein the measuring step employs hybridization to an allele-specific nucleic acid probe.

18. The method of claim 1 wherein the measuring step employs amplification on a bead in an emulsion.
19. The method of claim 18 wherein DNA fragments are amplified prior to amplification in an emulsion.
20. The method of claim 19 wherein DNA fragments are thermally denatured prior to hybridization to allele-specific nucleic acid probes, and cooled in the presence of tetramethyl ammonium chloride (TMAC).
21. The method of claim 20 wherein the cooling is at least as slow as 0.1 °C per second.
22. The method of claim 1 wherein the test sample is blood.
23. The method of claim 1 wherein the test sample is stool and the tumor is a colorectal tumor.
24. The method of claim 1 wherein the method is sufficiently sensitive to detect DNA fragments of a gene that have a mutation when said DNA fragments comprise 0.18% of the total number of said fragments of the gene.
25. The method of claim 1 wherein the method is sufficiently sensitive to detect DNA fragments of a gene that have a mutation when said DNA fragments comprise 0.005% of the total number of said fragments of the gene.
26. The method of claim 1 wherein number of copies of DNA fragments are measured and wherein the method of measuring segregates and assays individual DNA fragments.
27. The method of claim 1 wherein the DNA fragments of the gene that have the mutation are detected within 2 months of tumor resection.
28. The method of claim 1 wherein the DNA fragments of the gene that have the mutation are detected within 1 week of tumor resection.

29. The method of claim 1 wherein the DNA fragments of the gene that have the mutation are detected within 1 day of tumor resection.

30. The method of claim 1 wherein the DNA fragments of the gene that have the mutation are detected after 2 days post tumor resection.



↑
FROM FIG. 1A

DIRECT SEQUENCING

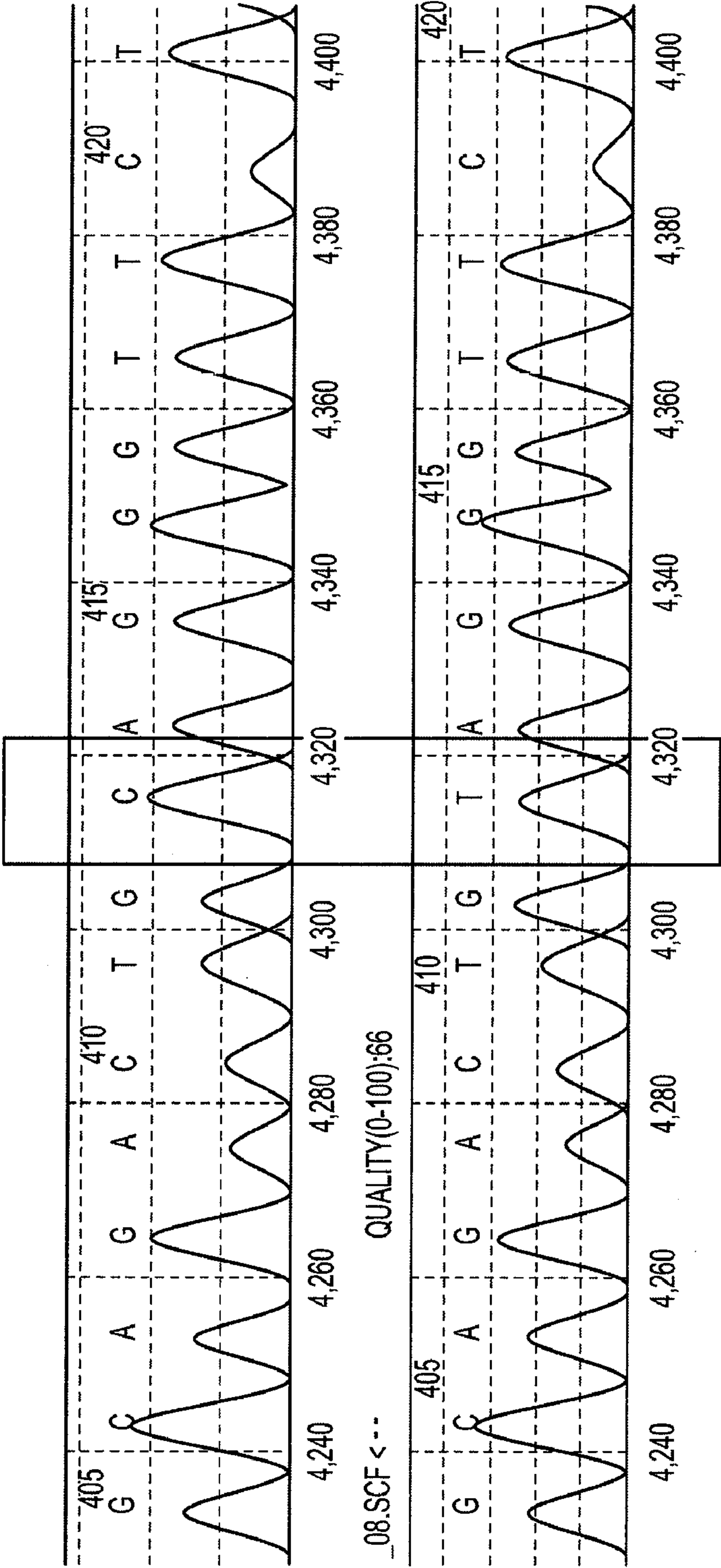
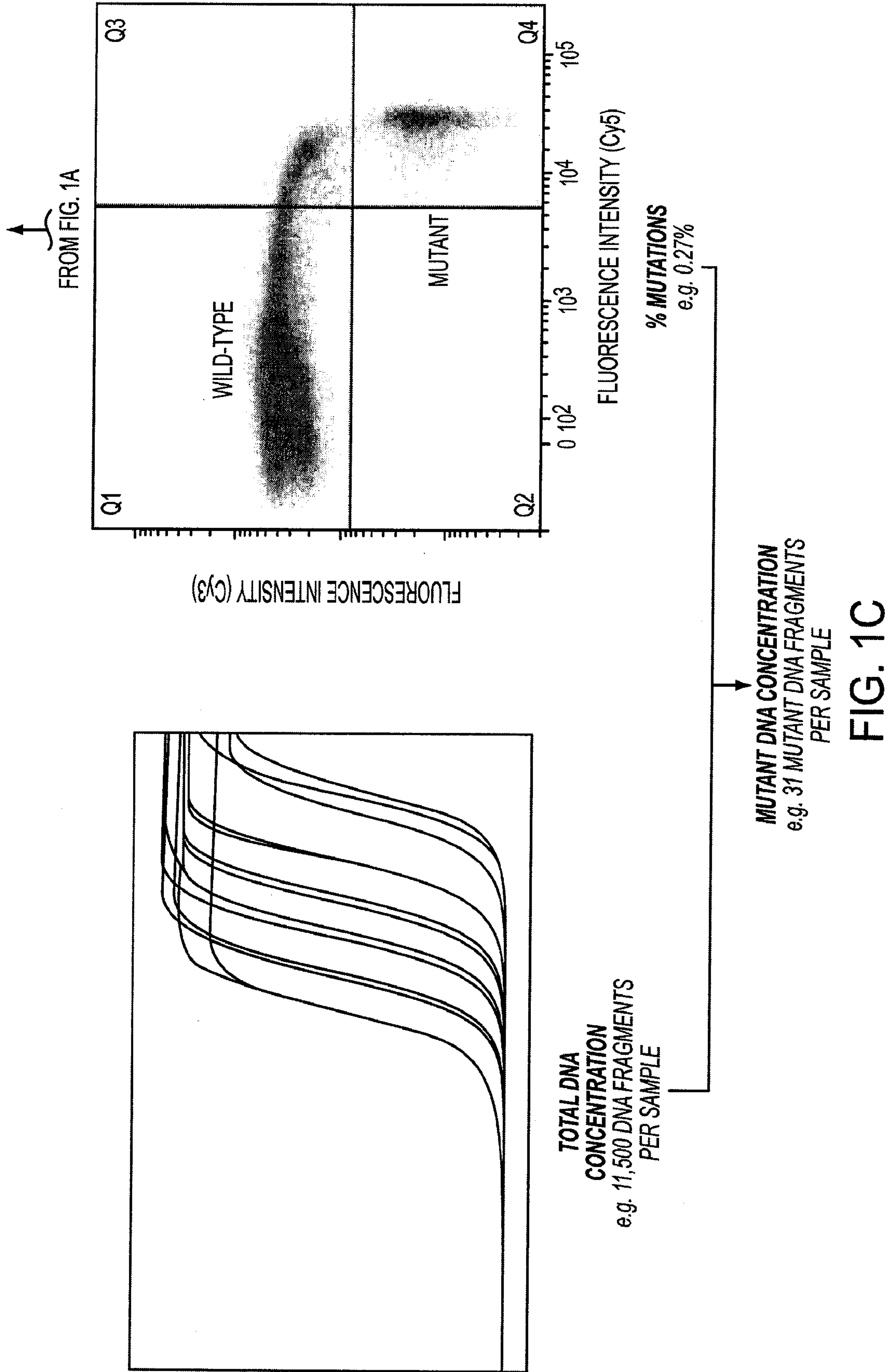


FIG. 1B

3/51



4/51

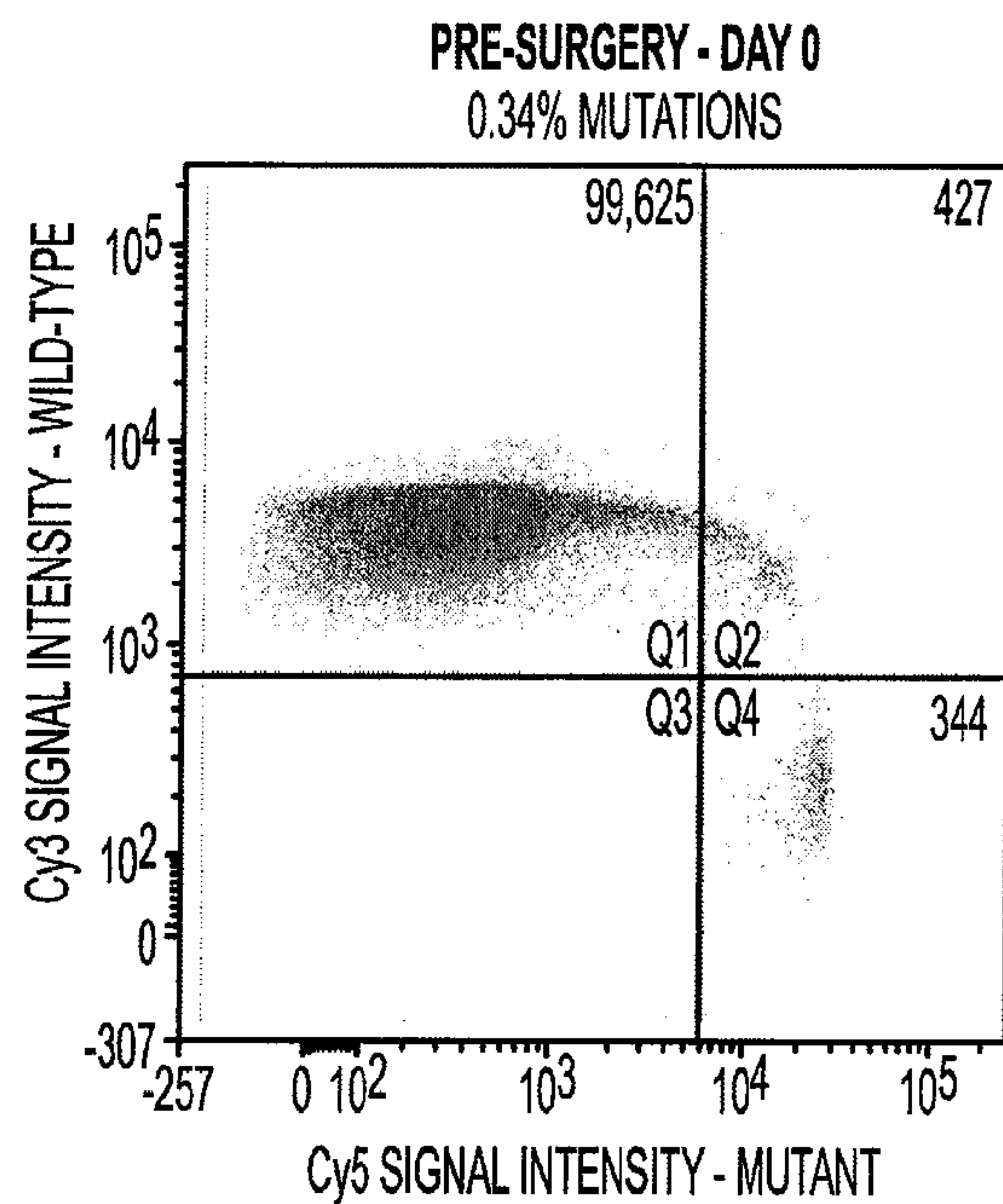


FIG. 2A

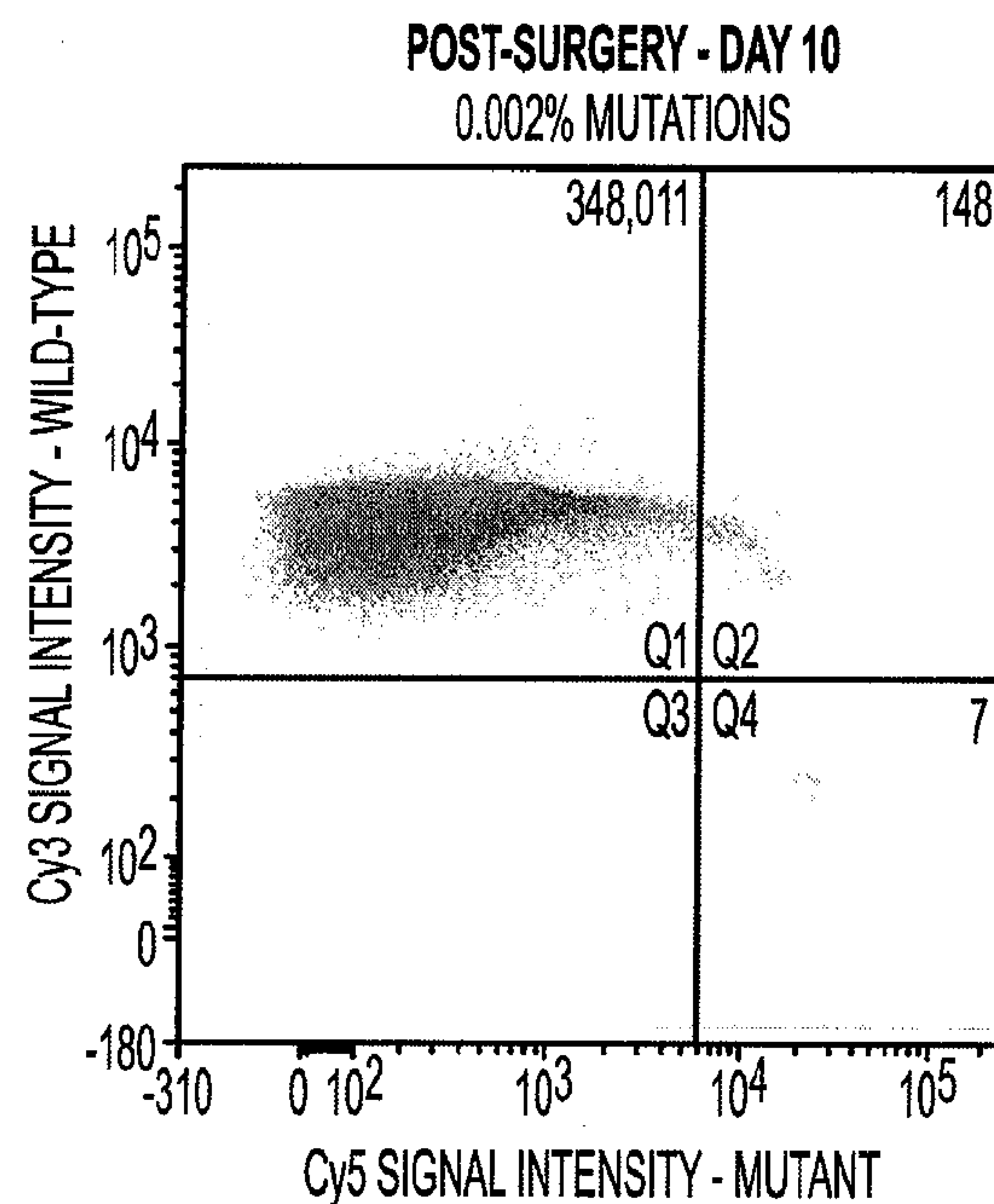


FIG. 2B

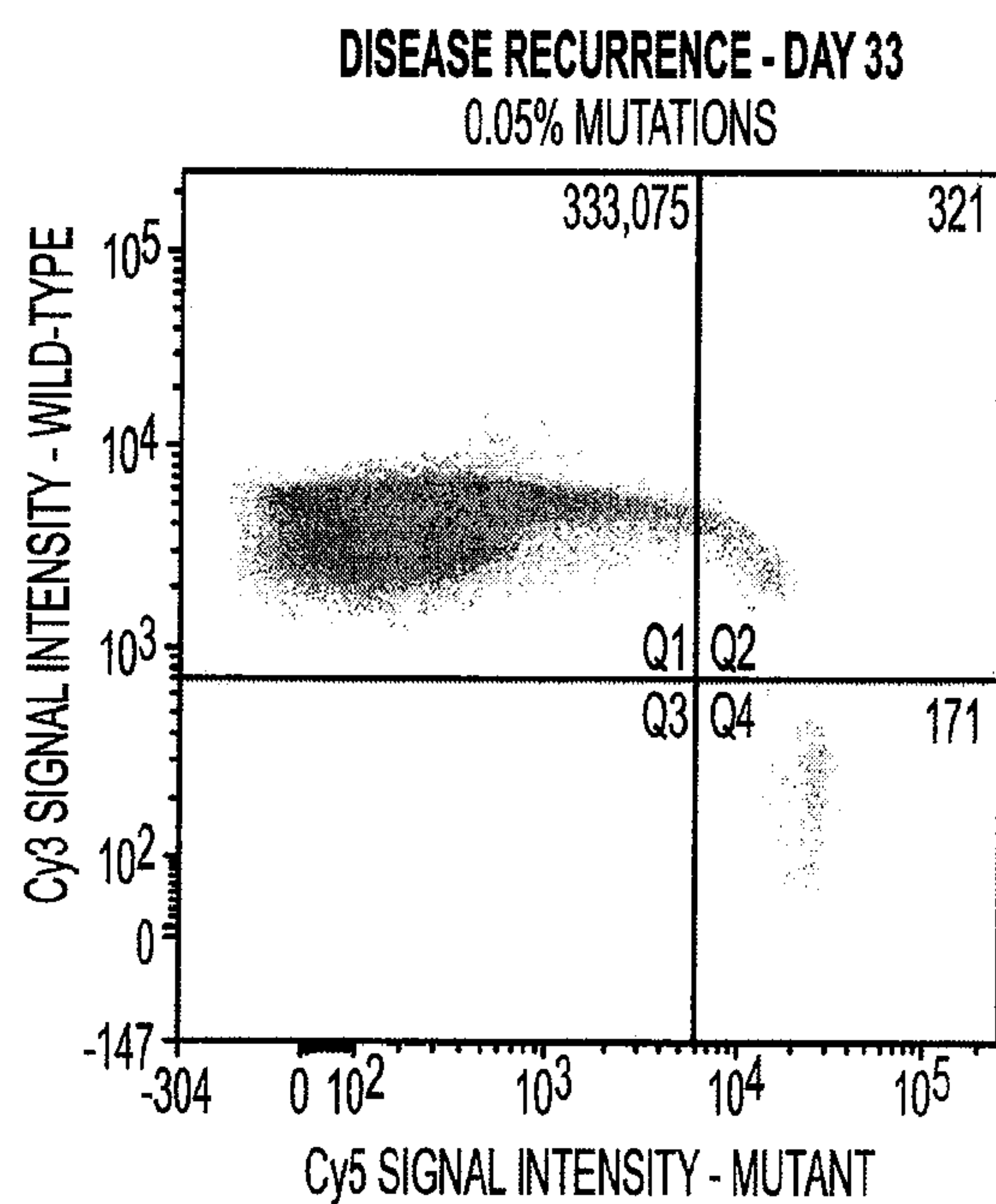


FIG. 2C

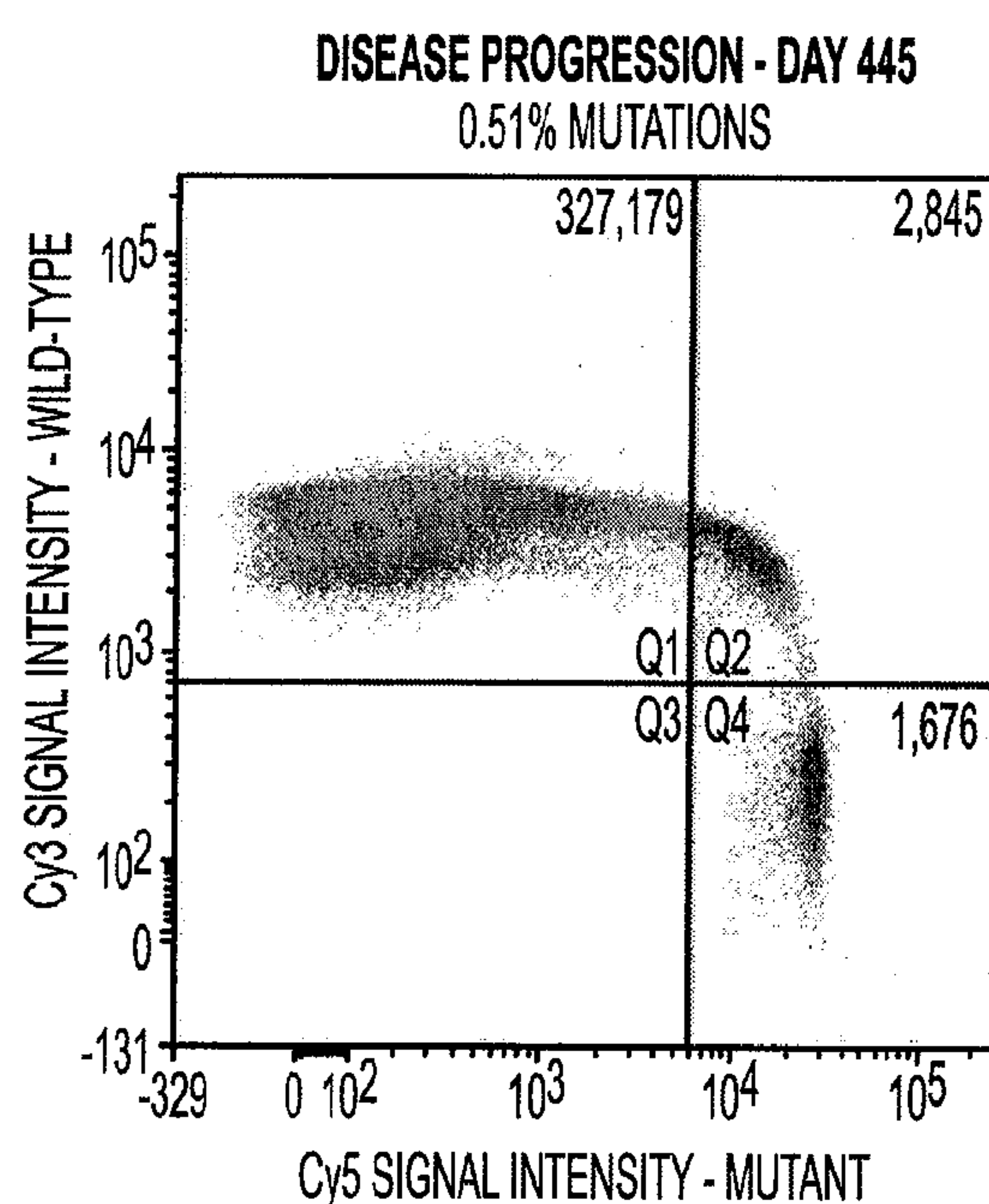


FIG. 2D

5/51

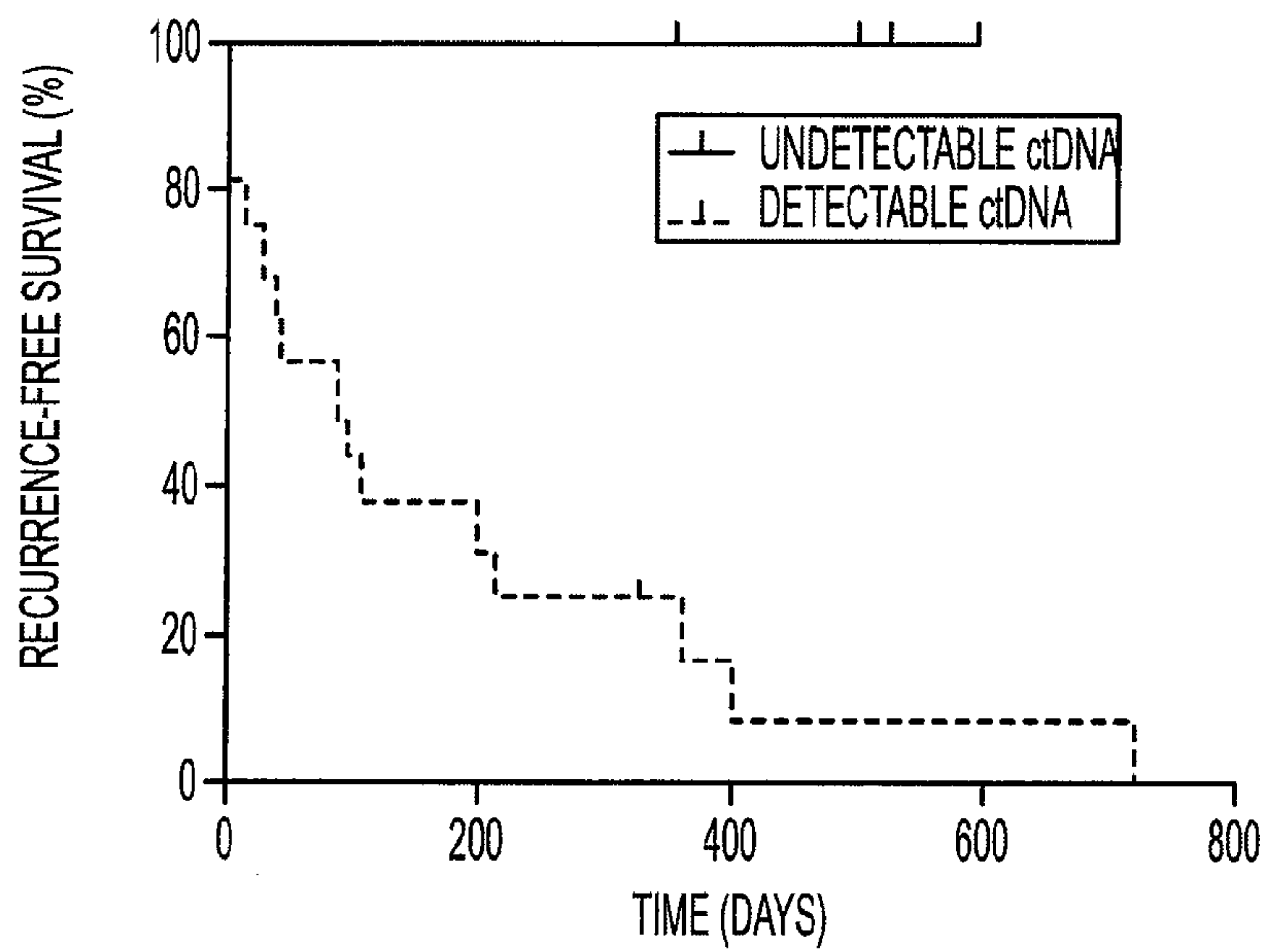


FIG. 3A

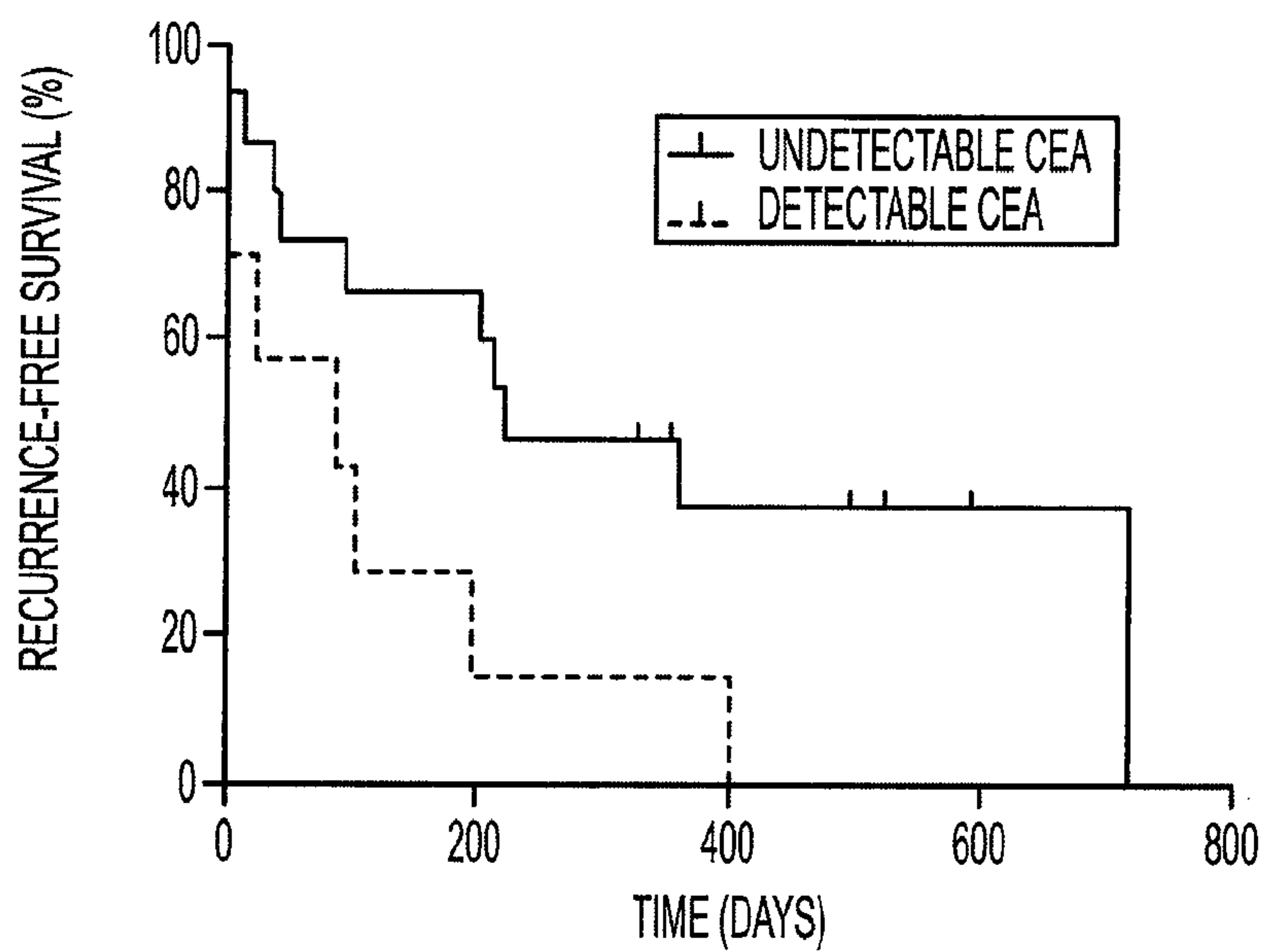


FIG. 3B

6/51

PATIENT 5

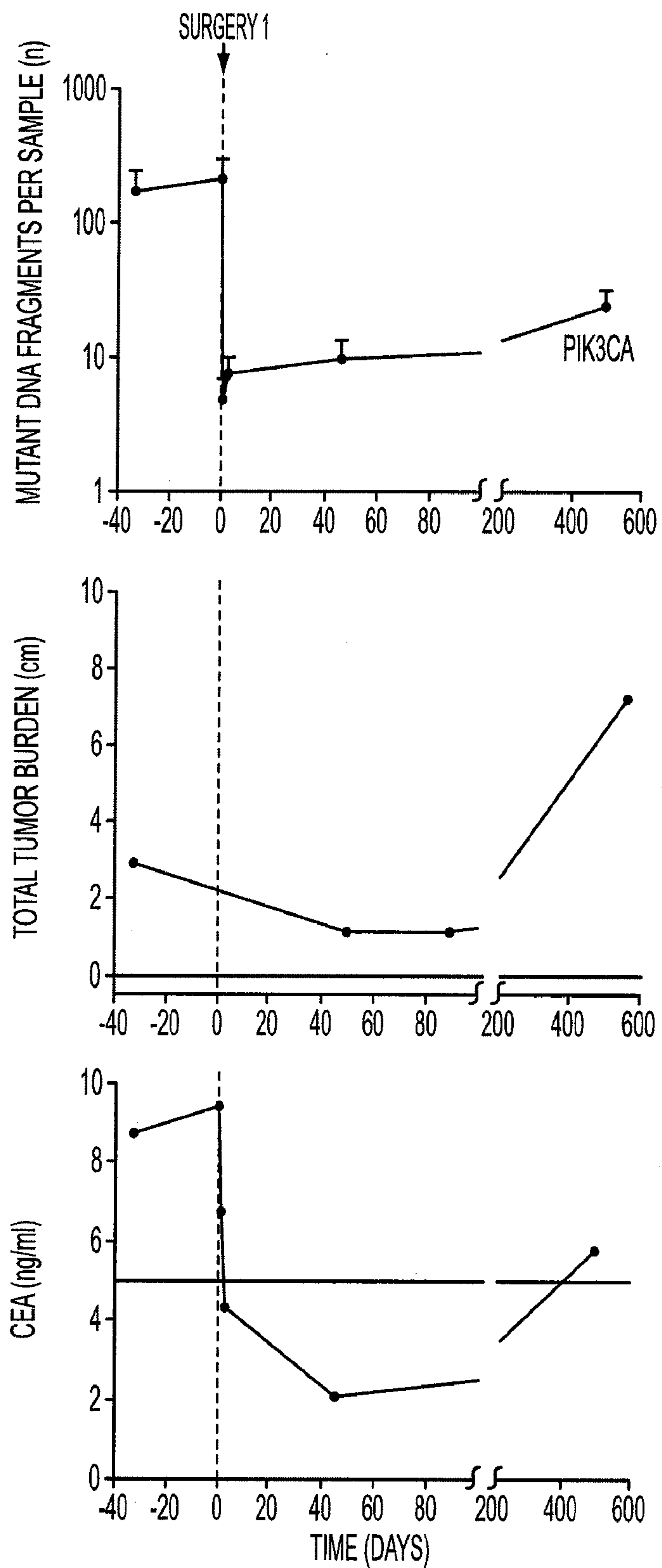


FIG. 4A

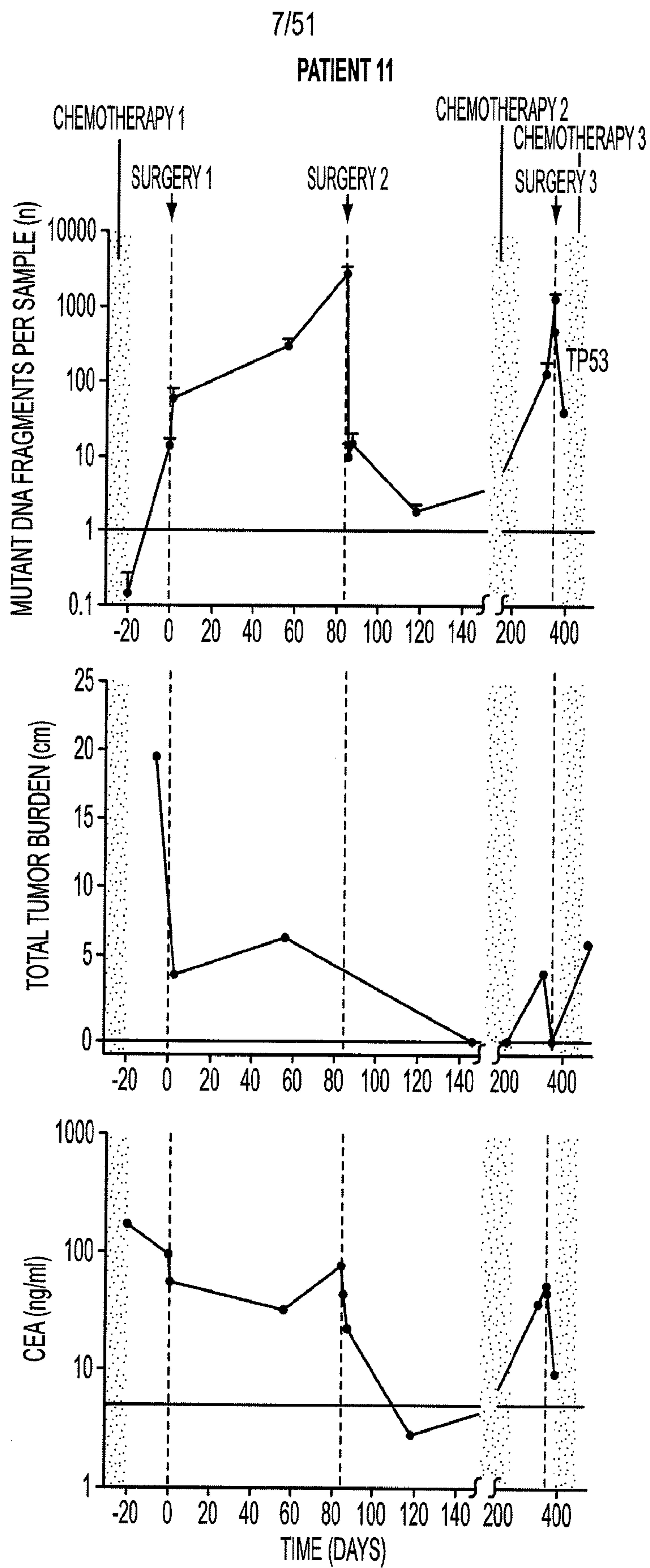


FIG. 4B

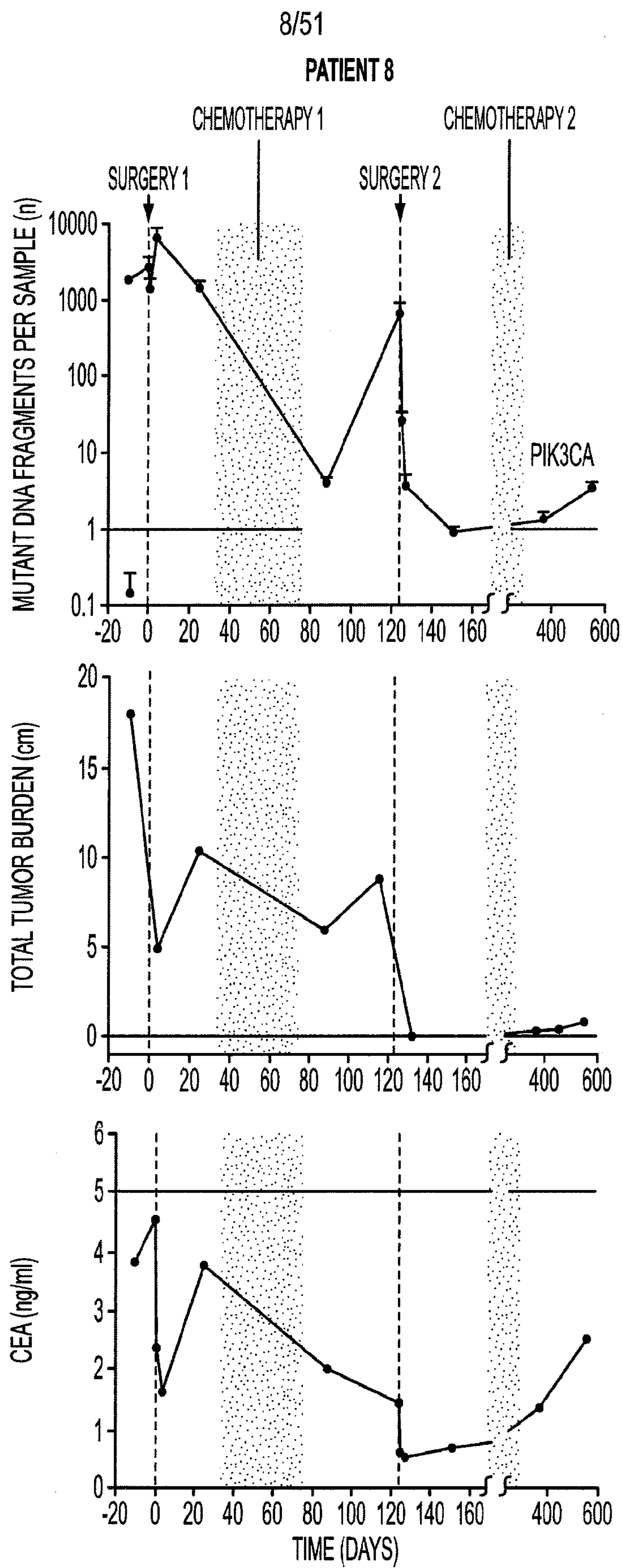


FIG. 4C

9/51

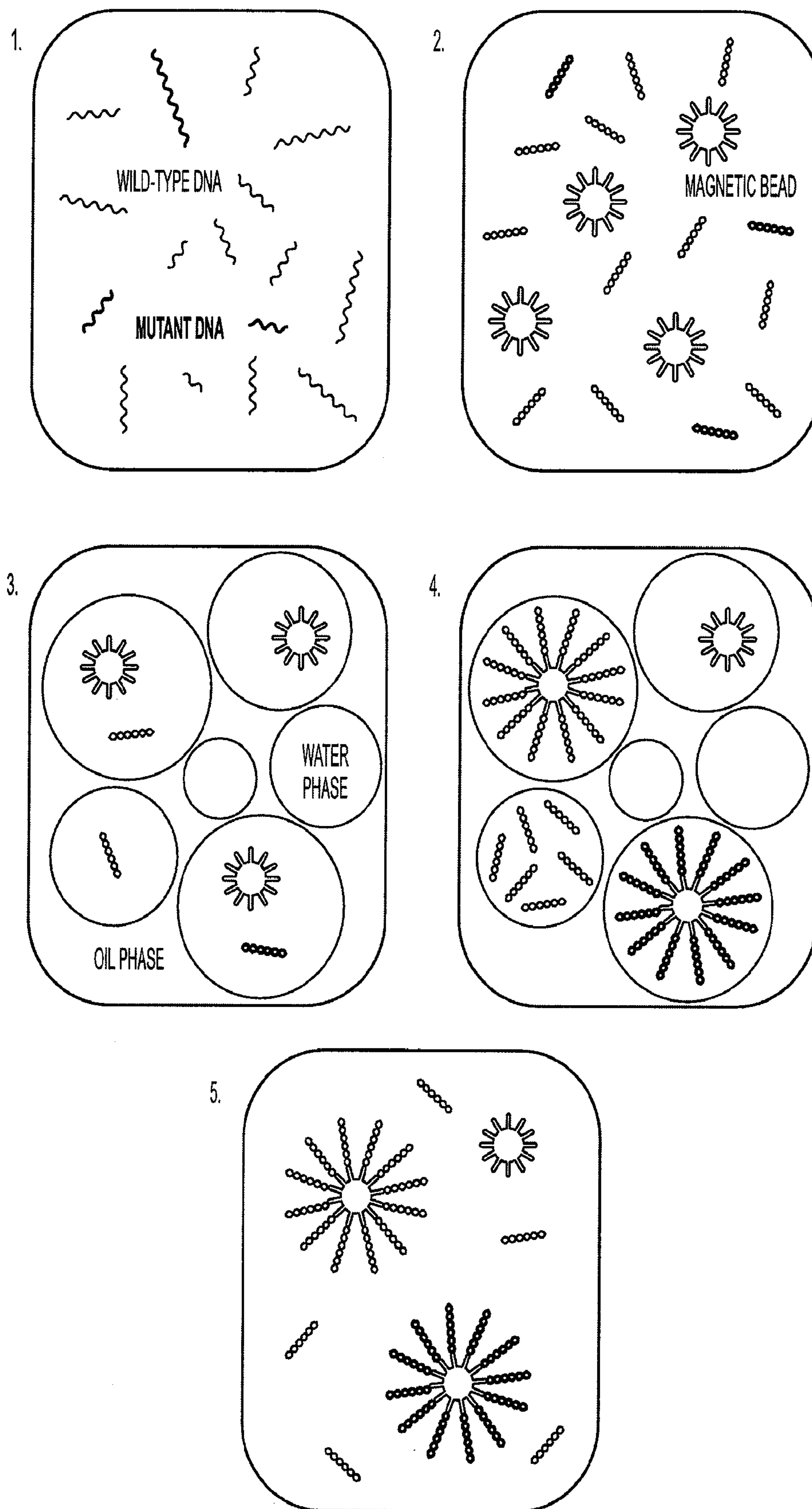


FIG. 5A

10/51

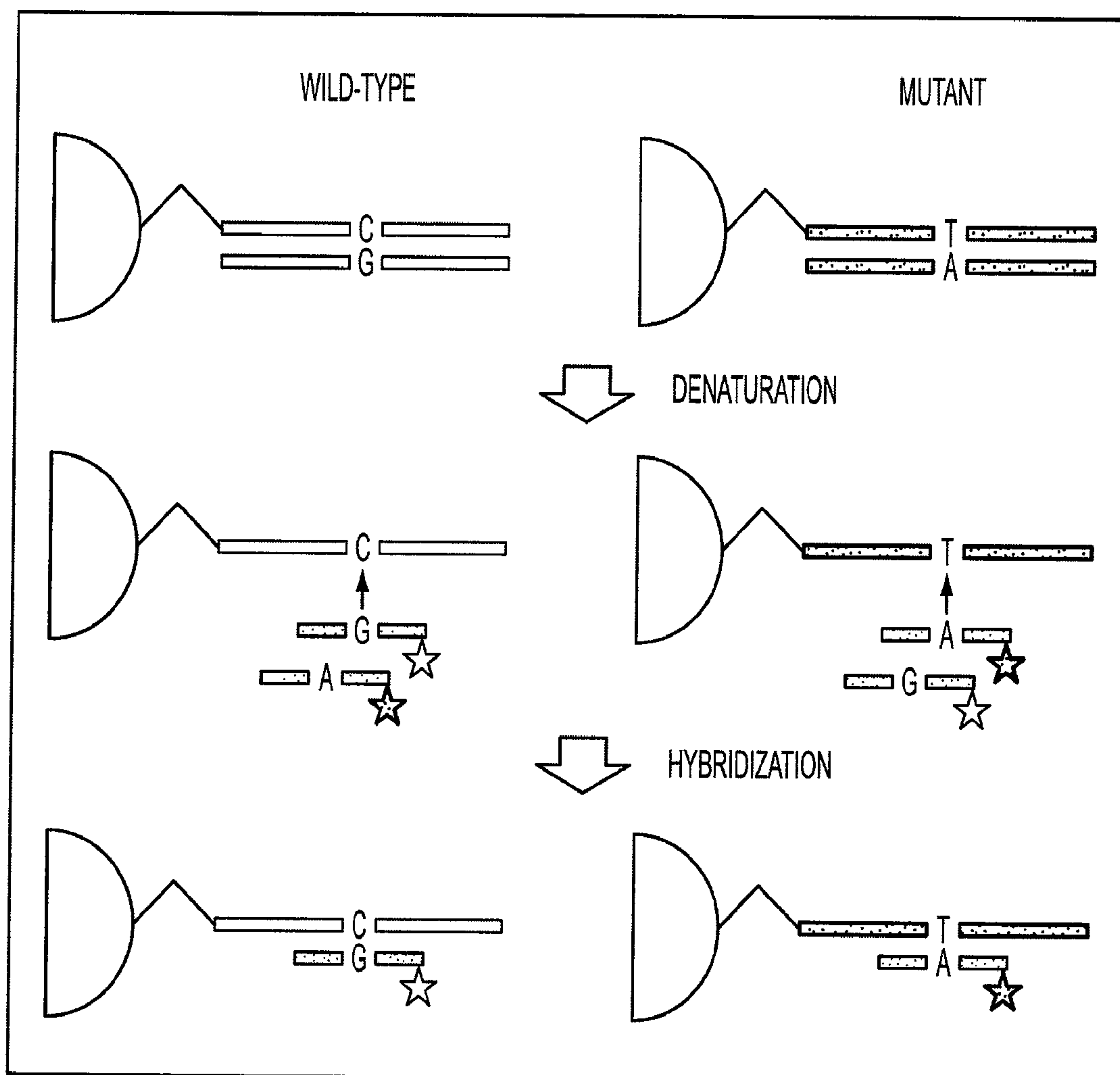


FIG. 5B

11/51

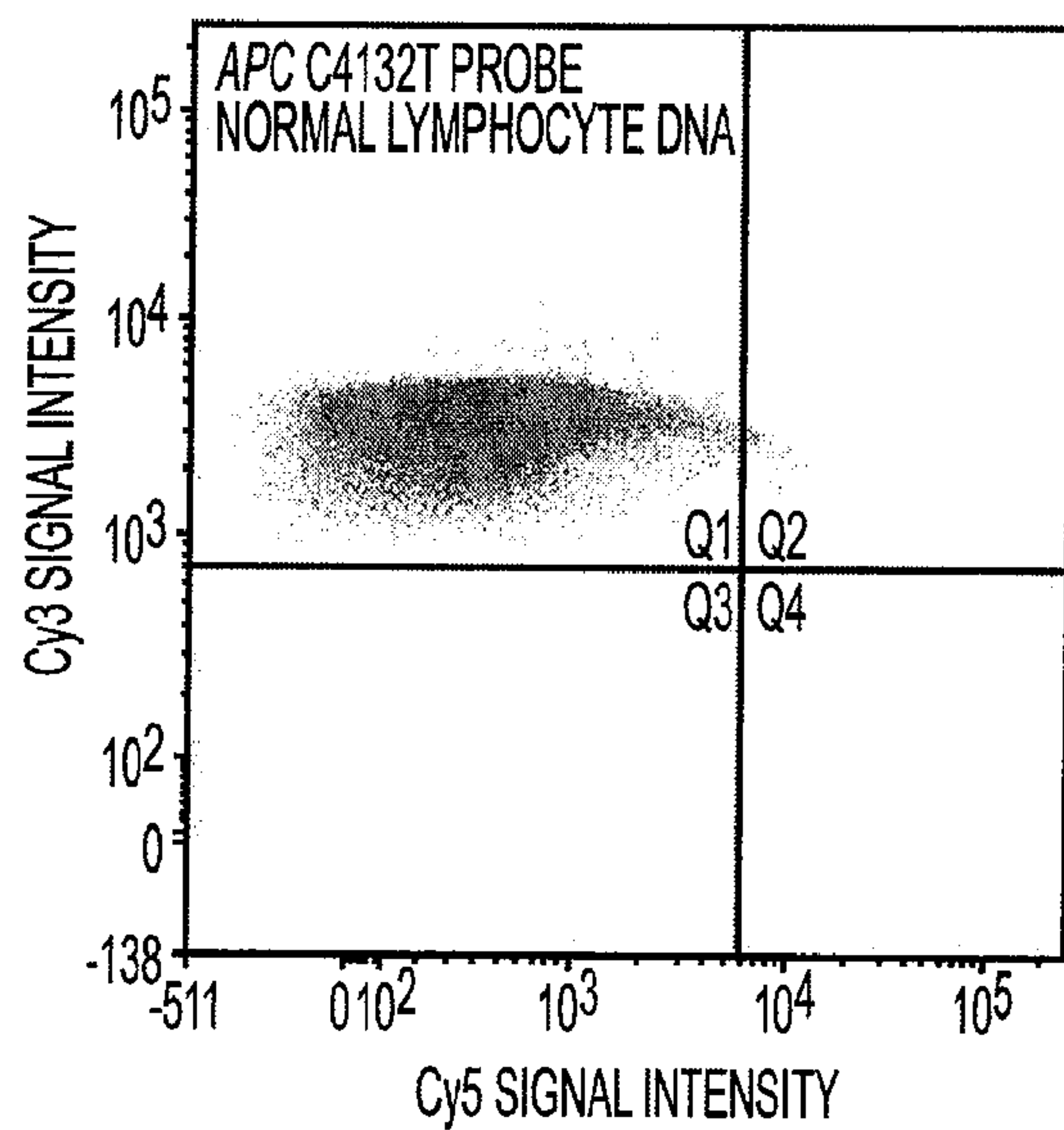


FIG. 6A

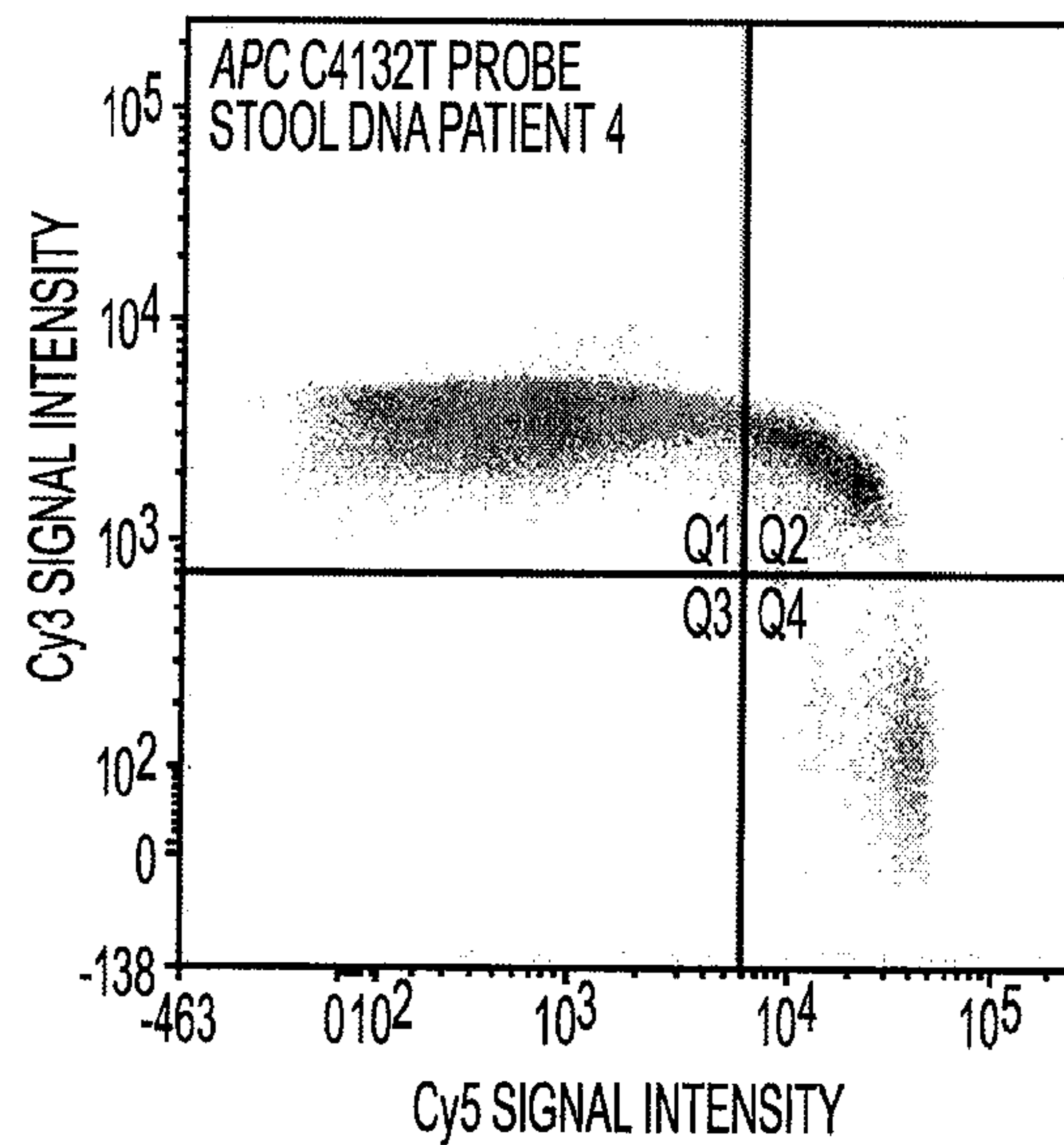


FIG. 6B

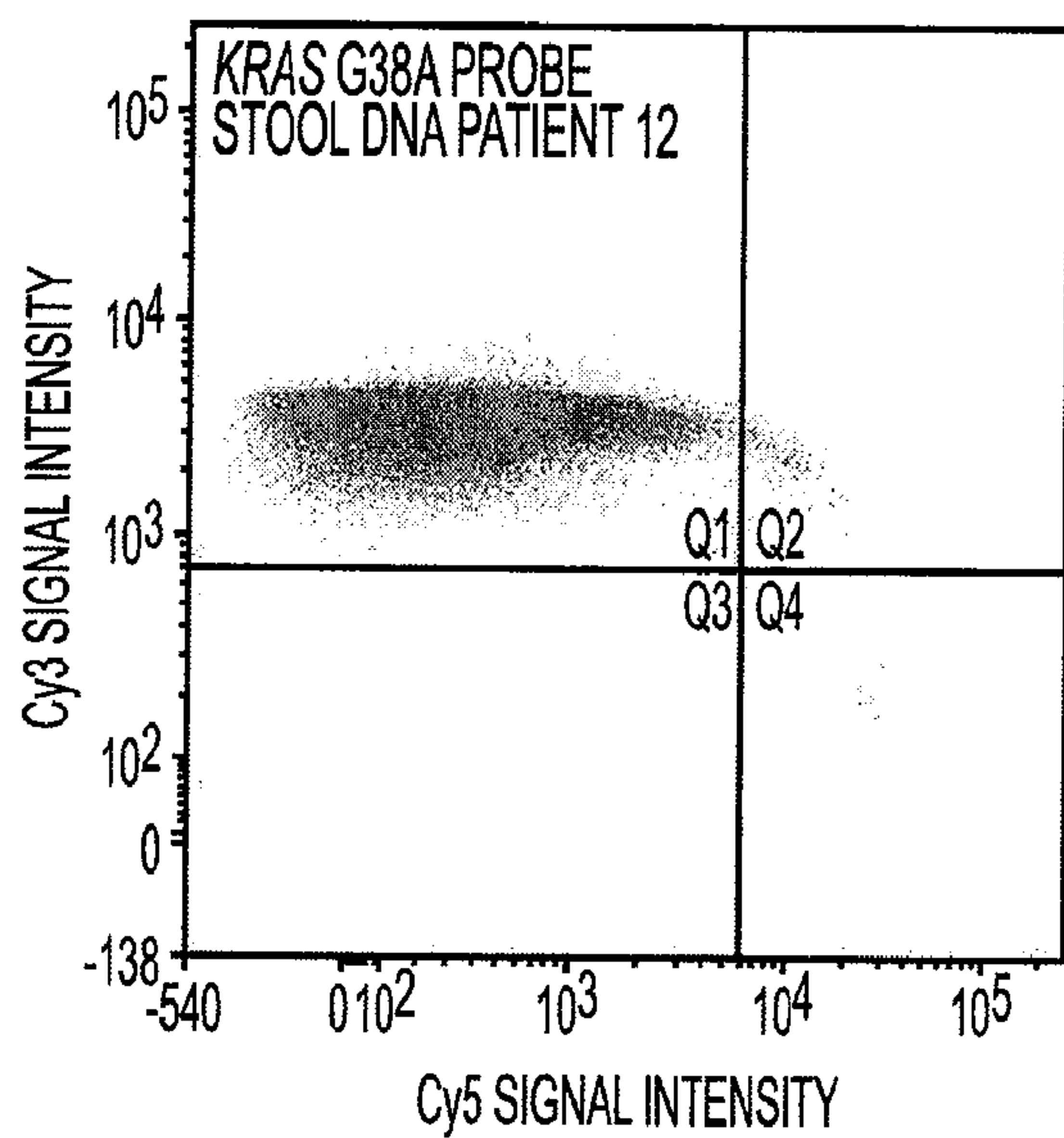


FIG. 6C

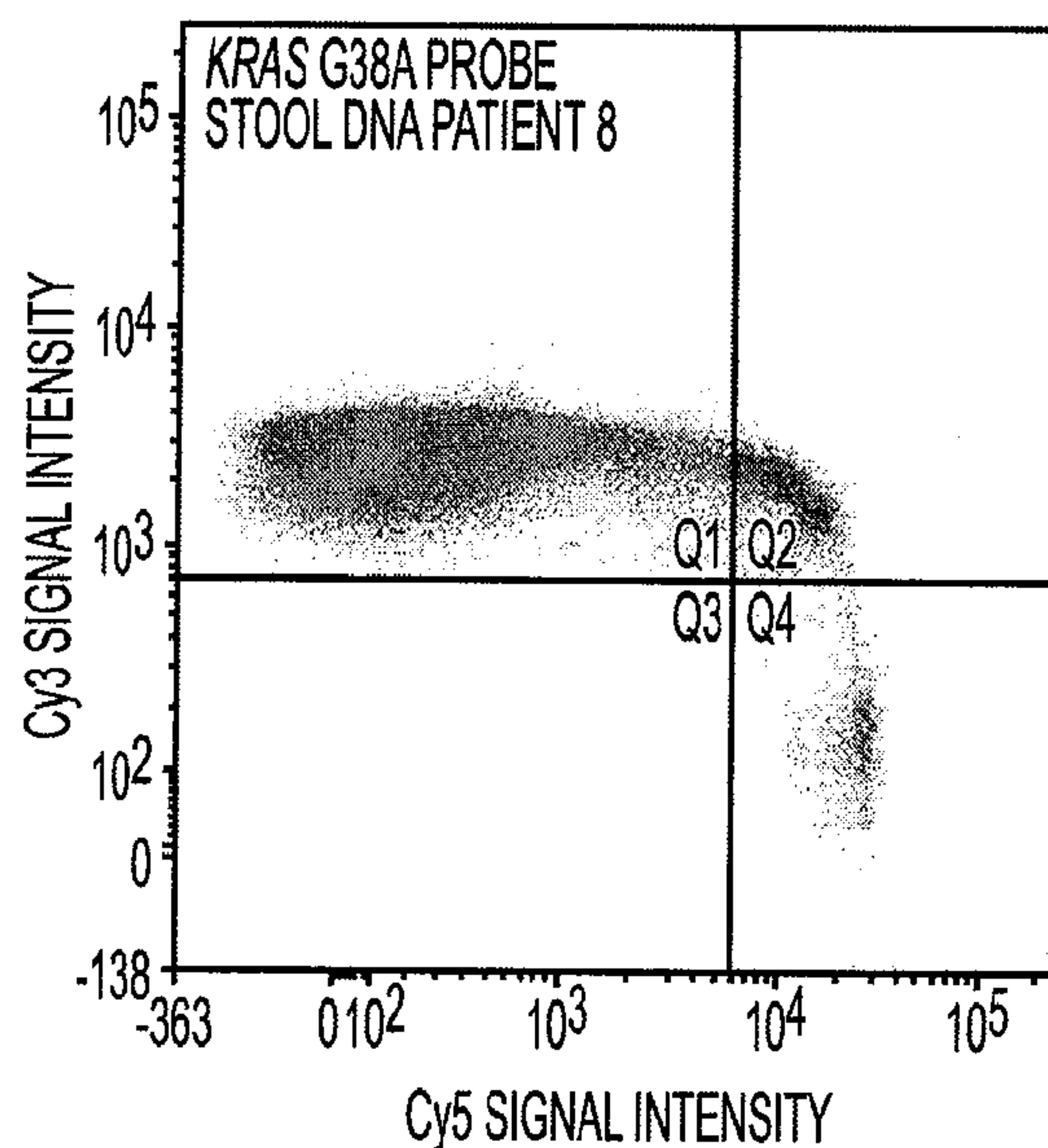


FIG. 6D

12/51

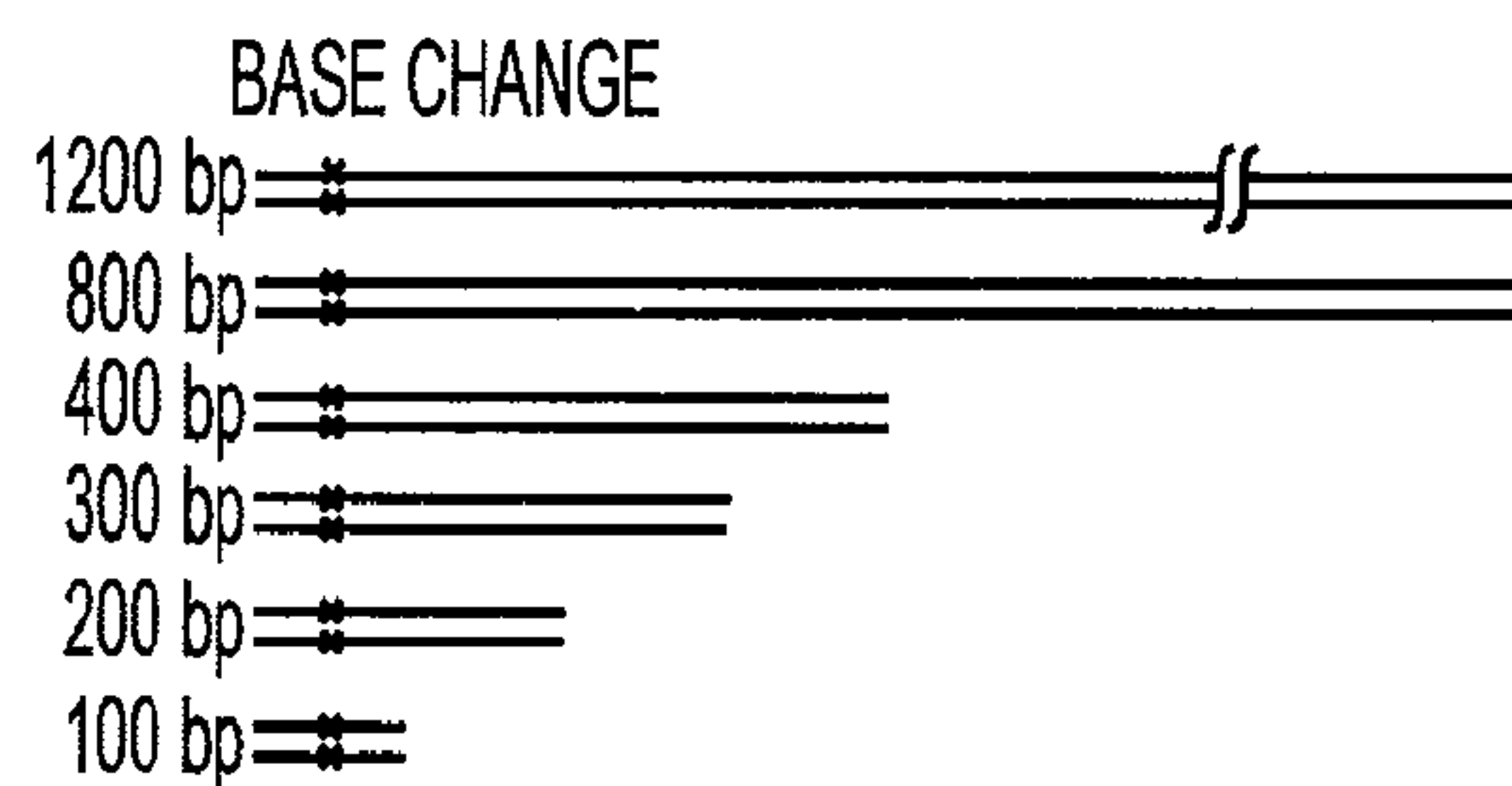


FIG. 7A

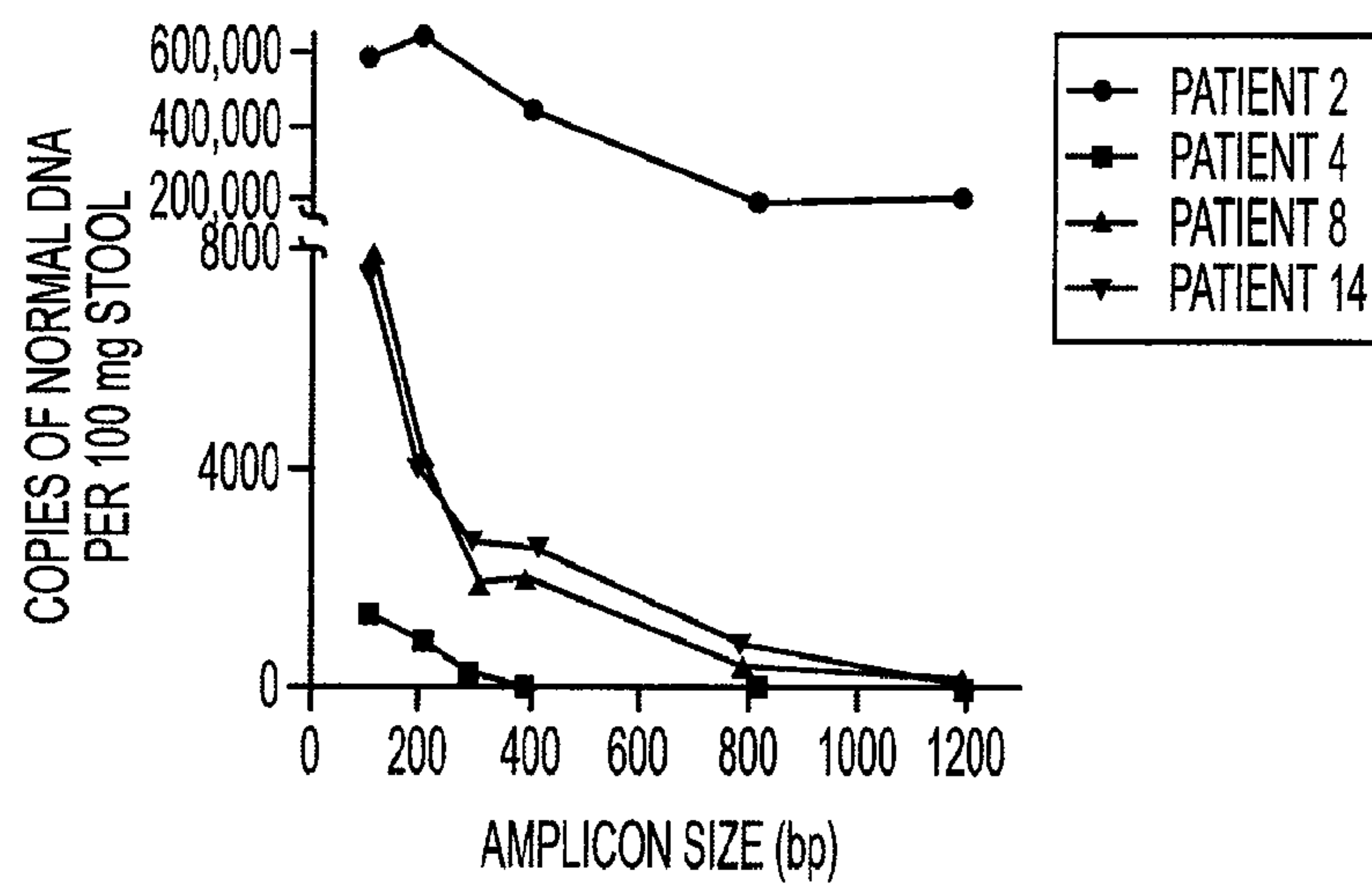


FIG. 7B

13/51

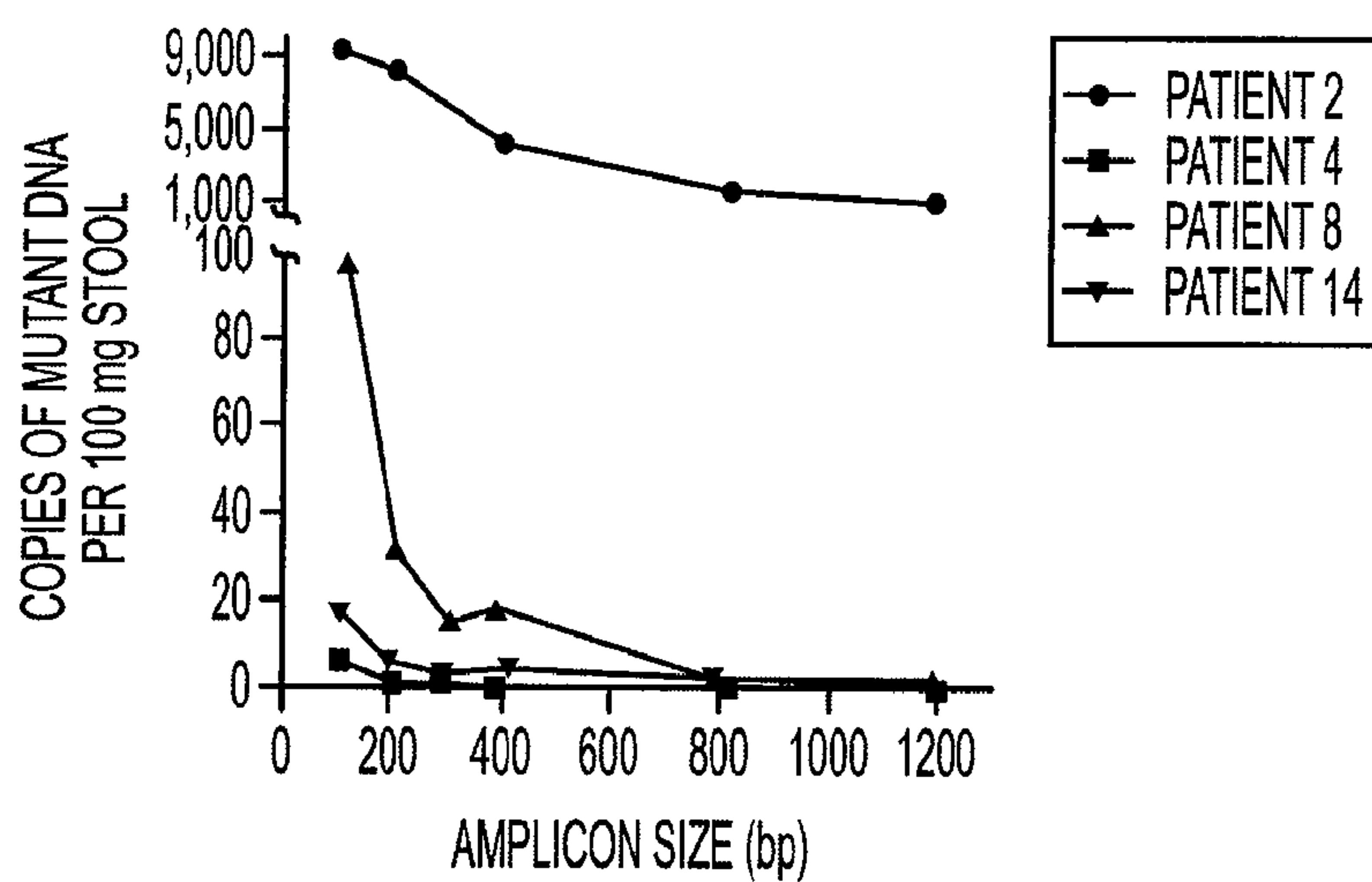


FIG. 7C

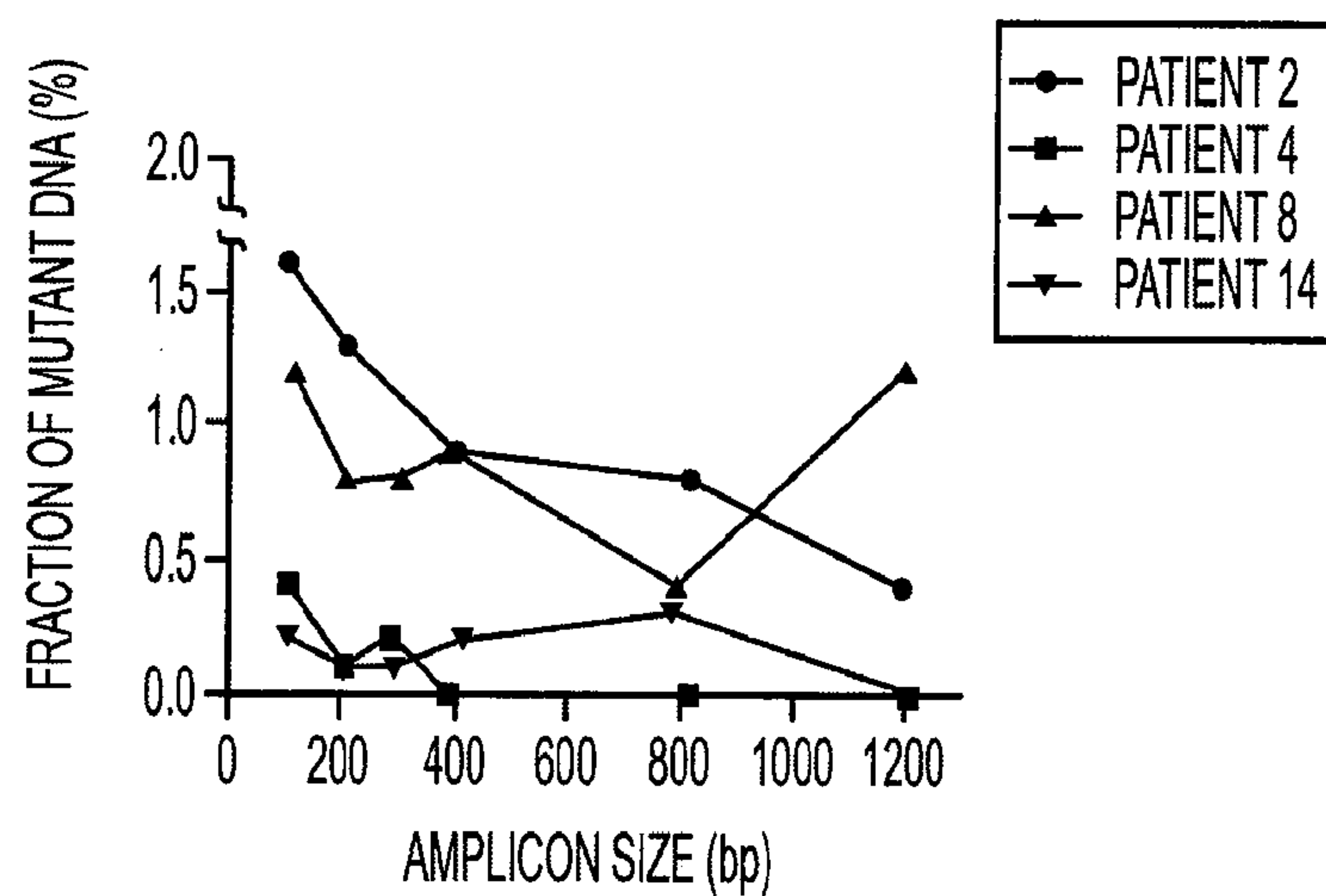


FIG. 7D

14/51

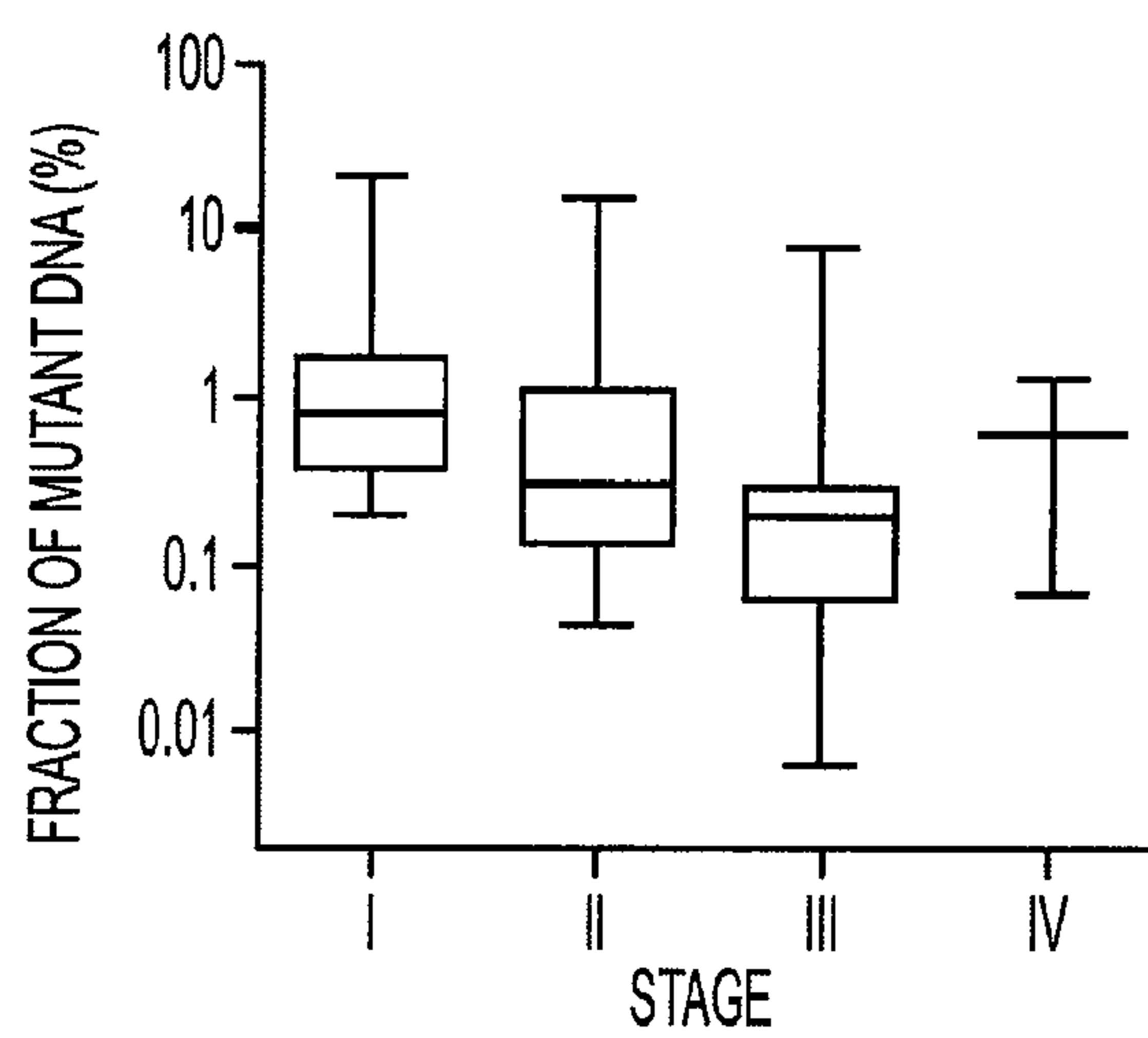


FIG. 8

15/51

AMPLICON	PATIENT	NUCLEOTIDE REGION	SIZE (bp)	PCR	FORWARD PRIMER 5'-3'	REVERSE PRIMER 5'-3'	PCR CONDITIONS ¹
APC - 1	1, 6, 13, 19	2555-2653	99	1ST	TAG2-tggagagagaacgcggaattggt	TAG1-ctgcagtggaggatctgcaaac	A
APC - 2	18, 25	3932-3981	98	1ST	TAG1-gaagcagattctgctgctaataccctgcaa	TAG2-tgacactgctggaacttgcctcac	A
APC - 3	2, 9, 22	3921-4016	96	1ST	aaaagaaaagattggaactaggctcagctgaaga	ccctgcagctcgtggaattgggt	A
		3921-4016	96	2ND	TAG1-aaaagaaaagattggaactaggctcagctgaaga	TAG2-ccctgcagctcgtggaattgggt	D
APC - 4	4, 12, 17	4092-4172	81	1ST	TAG2-tggtgctcagacacccaaaagtc	TAG1-ctgacagaaglacatctgctaaacatgagtgg	A
APC - 5	13, 24	4132-4246	115	1ST	TAG1-caggagacccactcatgttagcag	TAG2-cacttaccattccactgcatggttcac	A
APC - 6	3	4146-4295	100	1ST	TAG2-gttcgattgccagctccgttc	TAG1-ggcctggtttgtccagggtatc	A
APC - 7	7, 15	4238-4327	90	1ST	TAG2-tggttaagtggtattataagcccagtg	TAG1-gagggtggagggtgtttactctgc	A
APC - 8	14, 21	4283-4380	98	1ST	TAG2-gacaaacctgcccacaag	TAG1-agcagtaggtgtttatttttagg	C
APC - 9	5	4322-4430	109	1ST	TAG2-cacctcccaaacagctcaaacca	TAG1-tgaactgcagcattactgcagcttg	A
APC - 10	6, 8, 10	4418-4528	111	1ST	atgctgcagttcagaggggtccag	tcagagcactcaggctggatgaac	A
		4423-4528	106	2ND	TAG2-gcagttcagaggggtccaggtcttc	TAG1-tcagagcactcaggctggatgaac	D
APC - 11	11, 20	4620-4710	91	1ST	TAG2-gcagccctaaagaatcaaatgaaa	TAG1-atcatcatctgaatcatctaataggtc	C
KRAS	5, 8, 15, 17, 19, 20, 21, 22	2-97	96	1ST	TAG2-tgactgaatataaactgttgtagttg	TAG1-catatctgtccacaaatgattc	C
PIK3CA EXON 9	14, 17	335-435	101	1ST	gctcaaaagcaatttctacacgagatccct	cagagaatctccatttagcacttacctgtgac	A
		335-424	90	2ND	TAG1-gctcaaaagcaatttctacacgagatccct	TAG2-catttttagcacttacctgtgactccatagaaaatc	D
PIK3CA EXON 20-1	7	3048-3113	66	1ST	TAG2-ttgatgacattgcatacttcg	TAG1-actccaaagcctctgtctca	C
PIK3CA EXON 20-2	24	3081-3178	98	1ST	TAG1-cttagataaaaactgagcaagaggctttggag	TAG2- ggaagatccaatccattttgtgtcc	B
TP53 EXON 5	9, 16, 25	485-INTRON	84	1ST	TAG1-tctacaagcagtcacagcacatgacg	TAG2- gctgctcaccatcgctatctgagc	A
TP53 EXON 7	6, 18	INTRON-779	126	1ST	gggcctgtgttatctctcagggttg	agctctccagtgatgatggtgagg	A
		INTRON-779	126	2ND	TAG1-gggcctgtgttatctctcagggttg	TAG2-agctctccagtgatgatggtgagg	D
TP53 EXON 8	2, 19	783-877	95	1ST	TAG2-tggtaatctactggagcgaacagctt	TAG1-ctttcttgcggagattctcttctctg	A

TAG1: tcccgcgaattaatacgac

TAG2: gctggagctctgcagcta

¹PCR CONDITIONS:

A, 98°C, 30 s; 35x (98°C, 10 s; 71°C, 10 s)

B, 98°C, 30 s; 35x (98°C, 10 s; 69°C, 10 s)

C, 98°C, 30 s; 3x (98°C, 10 s; 70°C, 10 s; 72°C, 10 s); 3x (98°C, 10 s; 67°C, 10 s; 72°C, 10 s), 3x

D, 98°C, 30 s; 4x (98°C, 10 s; 71°C, 10 s)

FIG. 9

16/51

GENE	MUTATION	PATIENT	AMPLICON	PROBE	PROBE SEQUENCE 5'-3'
APC	C2626T	1, 6, 13, 19	APC - 1	UNIVERSAL WILD-TYPE MUTANT	ROX-agcaacagaaaatccagga Cy3-cttcaaagcgaggttg Cy5-cttcaaagtgaggttg
APC	3927-3931 del AAAGA	25	APC - 2	UNIVERSAL WILD-TYPE MUTANT	ROX-cttcgctcacaggatcttc Cy3-atcttttctttWtttctg ¹ Cy5-ttccaatctttWtttctg ¹
APC	3934delG	18	APC - 2	UNIVERSAL WILD-TYPE MUTANT	ROX-cttcgctcacaggatcttc Cy3-acctagttccaatcttt Cy5-gacctagttcaatcttt
APC	G3964T	2	APC - 3	UNIVERSAL WILD-TYPE MUTANT	ROX-cagtctgctggatttggt Cy3-tggaacttcgctcacag Cy5-tggaacttagctcacag
APC	3980-3983 delCAC	22	APC - 3	UNIVERSAL WILD-TYPE MUTANT	ROX-cagtctgctggatttggt Cy3-ggtgctgtgacactgc Cy5-aggggtgctacactgct
APC	C3980G	9	APC - 3	UNIVERSAL WILD-TYPE MUTANT	ROX-cagtctgctggatttggt Cy3-ggtgctgtgacactgc Cy5-ggtgctgtcacactgc
APC	4126-4127 insT	17	APC - 4	UNIVERSAL WILD-TYPE MUTANT	ROX-gctcagacacccaaaagtc Cy3-gaacactatgttcaggag Cy5-gaacactatgttcagga
APC	C4132T	4	APC - 4	UNIVERSAL WILD-TYPE MUTANT	ROX-gctcagacacccaaaagtc Cy3-cactatgttcaggagacc Cy5-cactatgtttaggagacc
APC	G4135T	12	APC - 4	UNIVERSAL WILD-TYPE MUTANT	ROX-gctcagacacccaaaagtc Cy3-tatgttcaggagacccac Cy5-tatgttcagtagacccac
APC	G4189T	24	APC - 5	UNIVERSAL WILD-TYPE MUTANT	ROX-aactgacagaagtacatct Cy3-acgactctcaaaactat Cy5-acgactctaaaaactat
APC	4189-4190 delGA	13	APC - 5	UNIVERSAL WILD-TYPE MUTANT	ROX-aactgacagaagtacatct Cy3-acgactctcaaaactat Cy5-aacgactcaaaactatc
APC	4237-4240 delATGG	3	APC - 6	UNIVERSAL WILD-TYPE MUTANT	ROX-aagccccagtgatcttcca Cy3-cagtggaatggtaagtg Cy5-tgcagtggaataagtggc

↓
TO FIG. 10A-2
FIG. 10A-1

17/51

FROM FIG. 10A-1



APC	C4285T	15	APC - 7	UNIVERSAL WILD-TYPE MUTANT	ROX-aagccccagtgatcttcca Cy3-ccctggacaaacctg Cy5-ccctggataaacctg
APC	4297delC	7	APC - 7	UNIVERSAL WILD-TYPE MUTANT	ROX-aagccccagtgatcttcca Cy3-catgccaccaagcaga Cy5-ccatgccacaagcaga
APC	C4348T	14, 21	APC - 8	UNIVERSAL WILD-TYPE MUTANT	ROX-cagaagtaaaacacctcca Cy3-aaaccaagcgagaagta Cy5-aaaccaagtgagaagta
APC	4359delT	5	APC - 9	UNIVERSAL WILD-TYPE MUTANT	ROX-cctactgctgaaaagagag Cy3-tgaagtacctaaaaataaa Cy5-tagaagtaccaaaaataaa
APC	4465delT	6	APC - 10	UNIVERSAL WILD-TYPE MUTANT	ROX-tcagaggggtccaggttctt Cy3-tactttattacatttgc Cy5-atactttatacatttgc
APC	4467delA	10	APC - 10	UNIVERSAL WILD-TYPE MUTANT	ROX-tcagaggggtccaggttctt Cy3-actttattacatttgc Cy5-tactttattcatttgc
APC	4497delA	8	APC - 10	UNIVERSAL WILD-TYPE MUTANT	ROX-tcagaggggtccaggttctt Cy3-ccagatggattttcttg Cy5-tccagatggttttcttg
APC	4661-4662 insA	11, 20	APC - 11	UNIVERSAL WILD-TYPE MUTANT	ROX-aaatgaaaaccaagagaaa Cy3-agaggcagaaaaaacta Cy5-agaggcagaaaaaaact

FIG. 10A-2

18/51

KRAS	G35A	5, 15 20, 21, 22	KRAS	UNIVERSAL WILD-TYPE MUTANT	ROX-tgacgatacagctaattca Cy3-ggagctggtggcgta Cy5-ggagctgatggcgta
KRAS	G35C	5, 19	KRAS	UNIVERSAL WILD-TYPE MUTANT	ROX-tgacgatacagctaattca Cy3-ggagctggtggcgta Cy5-ggagctgctggcgta
KRAS	G35T	5	KRAS	UNIVERSAL WILD-TYPE MUTANT	ROX-tgacgatacagctaattca Cy3-ggagctggtggcgta Cy5-ggagctgtggcgta
KRAS	G38A	8, 17	KRAS	UNIVERSAL WILD-TYPE MUTANT	ROX-tgacgatacagctaattca Cy3-tgctggtggcgtaggc Cy5-tgctggtgacgtaggc
PIK3CA	G1624A	14, 17	PIK3CA EXON 9	UNIVERSAL WILD-TYPE MUTANT	ROX-acctgtgactccatagaaa Cy3-agtgatttcagagagag Cy5-agtgattttagagagag
PIK3CA	C1636A	23	PIK3CA EXON 9	UNIVERSAL WILD-TYPE MUTANT	ROX-acctgtgactccatagaaa Cy3-ctttctcctgctcagtga Cy5-ctttctccttctcagtga
PIK3CA	C3075T	7	PIK3CA EXON 20 - 1	UNIVERSAL WILD-TYPE MUTANT	ROX-cttttgatgacattgcata Cy3-cgaaagaccctagcctt Cy5-cgaaagactctagcctt
PIK3CA	A3140G	24	PIK3CA EXON 20-2	UNIVERSAL WILD-TYPE MUTANT	ROX-ccaatccattttgtgtgc Cy3-caccatgatgtgcatca Cy5-caccatgacgtgcatca
TP53	G524A	16, 9, 25	TP53 EXON 5	UNIVERSAL WILD-TYPE MUTANT	ROX-gctatctgagcagcgctca Cy3-aggggcagcgccctcac Cy5-aggggcagtgccctcac
TP53	G733A	18	TP53 EXON 7	UNIVERSAL WILD-TYPE MUTANT	ROX-acacatgtagttgtagtgg Cy3-ttcatgccgcccatgc Cy5-ttcatgctgcccatgc
TP53	C742T	6	TP53 EXON 7	UNIVERSAL WILD-TYPE MUTANT	ROX-acacatgtagttgtagtgg Cy3-tggcctccggttcatgc Cy5-tggcctccagttcatgc
TP53	C817T	19	TP53 EXON 8	UNIVERSAL WILD-TYPE MUTANT	ROX-cctgggagagaccggcgca Cy3-tgaggtgctgtttgtg Cy5-tgaggtgtgtttgtg
TP53	G818A	2	TP53 EXON 8	UNIVERSAL WILD-TYPE MUTANT	ROX-cctgggagagaccggcgca Cy3-tgaggtgctgtttgtg Cy5-tgaggtgcatgtttgtg

¹W, MIXED BASE a AND t DUE TO SNP

FIG. 10B

19/51

PATIENT	GENE	MUTATION	APPLICATION	SIZE, bp	PRIMER, 5'-3'
2	APC	C3964T	1ST PCR FORWARD	105	ccctgcaaatagcagaaataaaa
				205	ccaataigtgttttcaagatgtagtcca
				290	aagtggtcagcctcaaaagg
				399	gacaaagcagtaaaaccgaaca
				815	tgagcaaagacaatcaaggaa
				1189	tcggaaaattcaaataggaca
			1ST PCR REVERSE		TAG1-tgctggatttggttctaggg
			EMULSION PCR		ttggaactaggtcagctgaaga
4	APC	C4132T	1ST PCR FORWARD	105	ccaaaagtgggtgctcagaca
				203	ccctagaaccaaatccagca
				288	ccctgcaaatagcagaaataaaa
				389	tccaataigtgttttcaagatgtagttc
				815	aagccagtccttgtgtcaagaa
				1197	gtcaatacccagccgacctta
			1ST PCR REVERSE		TAG1-caaaactatcaagtgaactgacagaa
			EMULSION PCR		gctcagacacccaaaagtc
7	APC	4297delC	1ST PCR FORWARD	114	ccatgcagtggaatggtaag
				207	caggagacccactcatgtt
				307	agaatcagccaggcacaaaag
				391	tgaagatcctgtgagcgaag
				790	tgccacagatattccttcac
				1193	gggcaagacccaaacacata
			1ST PCR REVERSE		TAG1-agctgtttgaggaggtggtg
			EMULSION PCR		cttcagatagccctggaca
14	APC	C4348T	1ST PCR FORWARD	104	gccttgacaaacctgc
				195	tttgagagtcgttcgattgc
				296	ccaaaagtgggtgctcagaca
				413	ttccagcagtgctcacagcac
				786	aagagttcatctggacaaagca
				1192	tgagcaaagacaatcaaggaa
			1ST PCR REVERSE		TAG1-agcagtaggtgctttatttttagg
			EMULSION PCR		gacaaacctgccaccaag
Tag1, tcccgcgaaattaatcgcac					

FIG. 11

20/51

STAGE	PATIENTS (n=25)	MUTATIONS (n=45)
I	6/7	10/12
II	7/7	9/11
III	7/8	16/17
VI	2/2	3/3
UNKNOWN	1/1	2/2
TOTAL	23/25 (92%)	41/45 (92%)

FIG. 12

21/51

PATENT	GENE	MUTATION (CODON)	TUMOR DNA STATUS		STOOL DNA STATUS		
			SBE ¹	SEQUENCING ²	SBE ³	SEQUENCING ²	BEAMING
1	APC	C2626T (876)	POS	-	POS	-	POS
2	APC	G3964T (1322)	-	POS	-	POS	POS
	TP53	G818A (273)	POS	-	POS	-	POS
3	APC	4237-4240delATGG (1413)	-	POS	-	NEG	POS
4	APC	C4132T (1378)	POS	POS	POS	POS	POS
5	APC	4359delT (1453)	-	POS	-	NEG	NEG
	KRAS	G35X (12) ⁴	POS	-	NEG	-	NEG
6	APC	C2626T (876)	POS	-	POS	-	POS
	APC	4465delT (1489)	-	POS	-	NEG	POS
	TP53	C742T (248)	POS	-	POS	-	POS
7	APC	4297delC (1433)	-	POS	-	POS	POS
	PIK3CA	C3075T (1025)	-	POS	-	POS	POS
8	APC	4497delA (1499)	-	POS	-	NEG	NEG
	KRAS	G38A (12)	POS	-	NEG	-	POS
9	APC	C3980G (1327)	-	POS	-	NEG	POS
	TP53	G524A (175)	NEG	-	POS	-	POS
10	APC	4467 delA (1489)	-	POS	-	POS	POS
11	APC	4661-4662insA (1554)	POS	-	POS	-	POS
12	APC	G4135T (1379)	POS	POS	POS	POS	POS
13	APC	C2626T (876)	POS	-	POS	-	POS
	APC	4189-4190delGA (1397)	-	POS	-	NEG	POS
14	APC	C4348T (1450)	POS	POS	POS	POS	POS
	PIK3CA	G1624A (542)	-	POS	-	NEG	POS
15	APC	C4285T (1429)	-	POS	-	NEG	POS
	KRAS	G35A (12)	POS	-	NEG	-	POS
16	TP53	G524A (175)	POS	-	NEG	-	NEG

↓
TO FIG. 13B

FIG. 13A

22/51

FROM FIG. 13A



17	APC	4126-4127insT (1376)	-	POS	-	NEG	POS
	KRAS	G38A (12)	POS	-	NEG	-	POS
	PIK3CA	G1624A (542)	-	POS	-	NEG	POS
18	APC	3934delG (1312)	POS	POS	POS	NEG	POS
	TP53	G733A (245)	POS	-	POS	-	POS
19	APC	C2626T (876)	POS	-	NEG	-	POS
	KRAS	G35C (12)	POS	-	NEG	-	POS
	TP53	C817T (273)	POS	-	NEG	-	POS
20	APC	4661-4662insA (1554)	POS	-	NEG	-	POS
	KRAS	G35A (12)	POS	-	NEG	-	POS
21	APC	C4348T (1450)	POS	POS	NEG	NEG	POS
	KRAS	G35A (12)	POS	-	NEG	-	POS
22	APC	3980-3983delCAC (1327)	-	POS	-	POS	POS
	KRAS	G35A (12)	POS	-	POS	-	POS
23	PIK3CA	C1636 (546)	-	POS	-	NEG	POS
24	APC	G4189T (1397)	-	POS	-	NEG	POS
	PIK3CA	A3140G (1047)	-	POS	-	POS	POS
25 ⁵	APC	3927-3931 del AAAGA (1309)	-	-	POS	-	POS
	TP53	G524A (175)	-	-	POS	-	POS

POS, POSITIVE ASSAY; NEG, NEGATIVE ASSAY; -, NOT ANALYZED

¹SINGLE BASE EXTENSION ACCORDING TO WHITNEY *et al.* (REF. 4)²SEQUENCING ACCORDING TO KANN *et al.* (REF. 5)³SINGLE BASE EXTENSION ACCORDING TO WHITNEY *et al.* (REF. 4), REPORTED IN STUDY BY IZKOWITZ ET AL (REF. 7)⁴G35X MEANS G35A, G35C, OR G35T (SPECIFIC BASE CHANGE NOT DETERMINED)⁵TUMOR TISSUE NOT RECEIVED

FIG. 13B

23/51

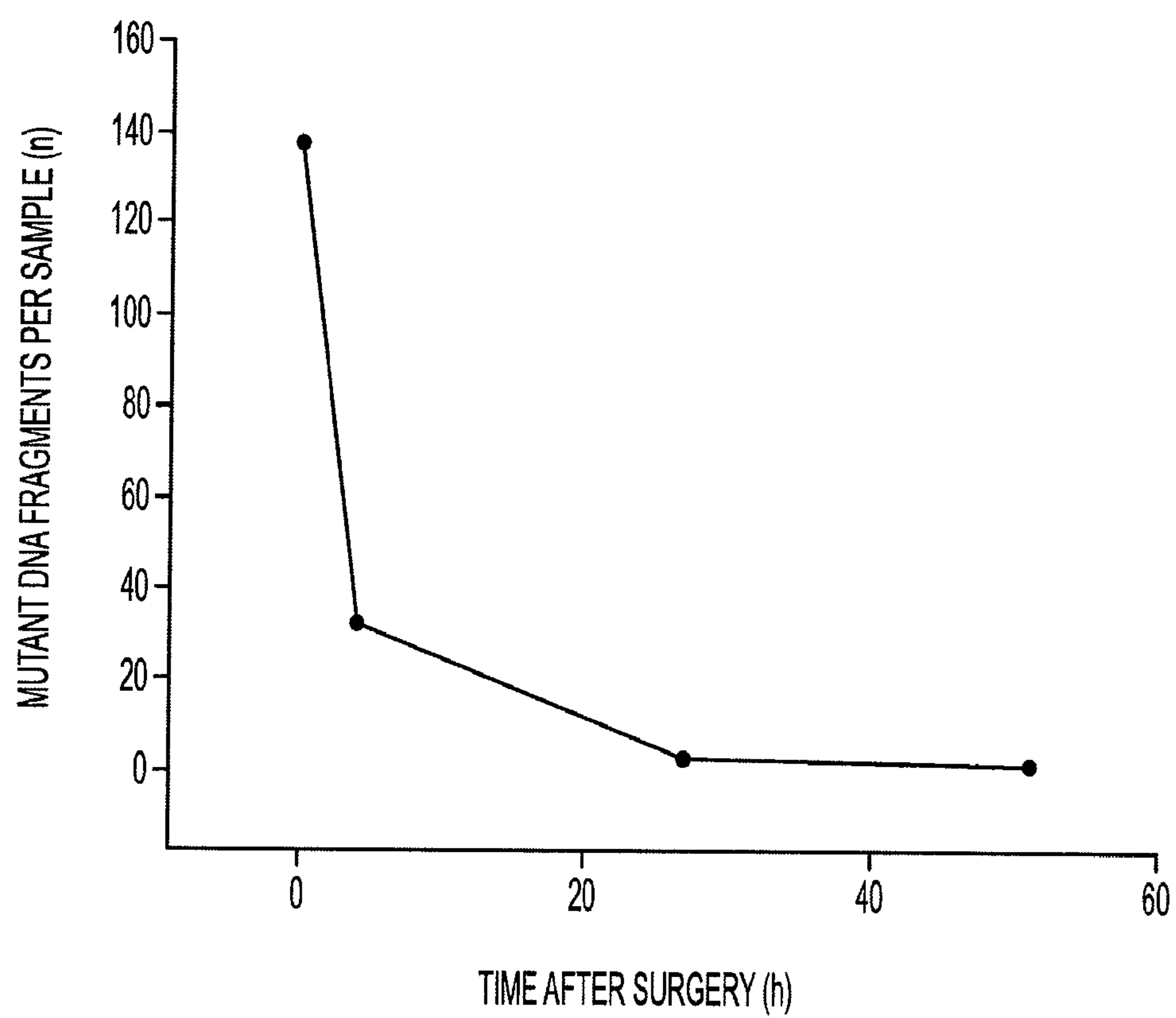


FIG. 14

24/51

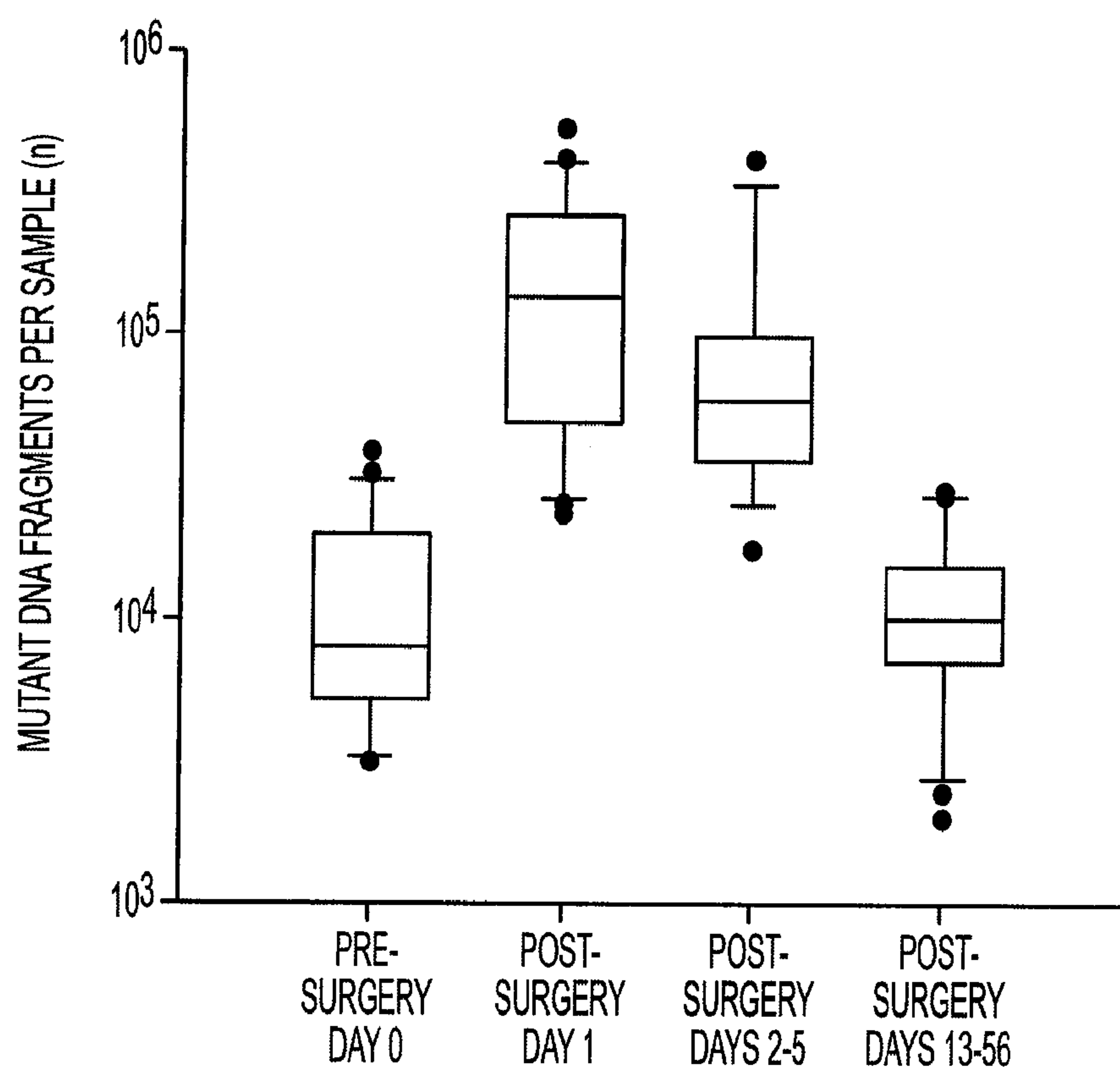


FIG. 15

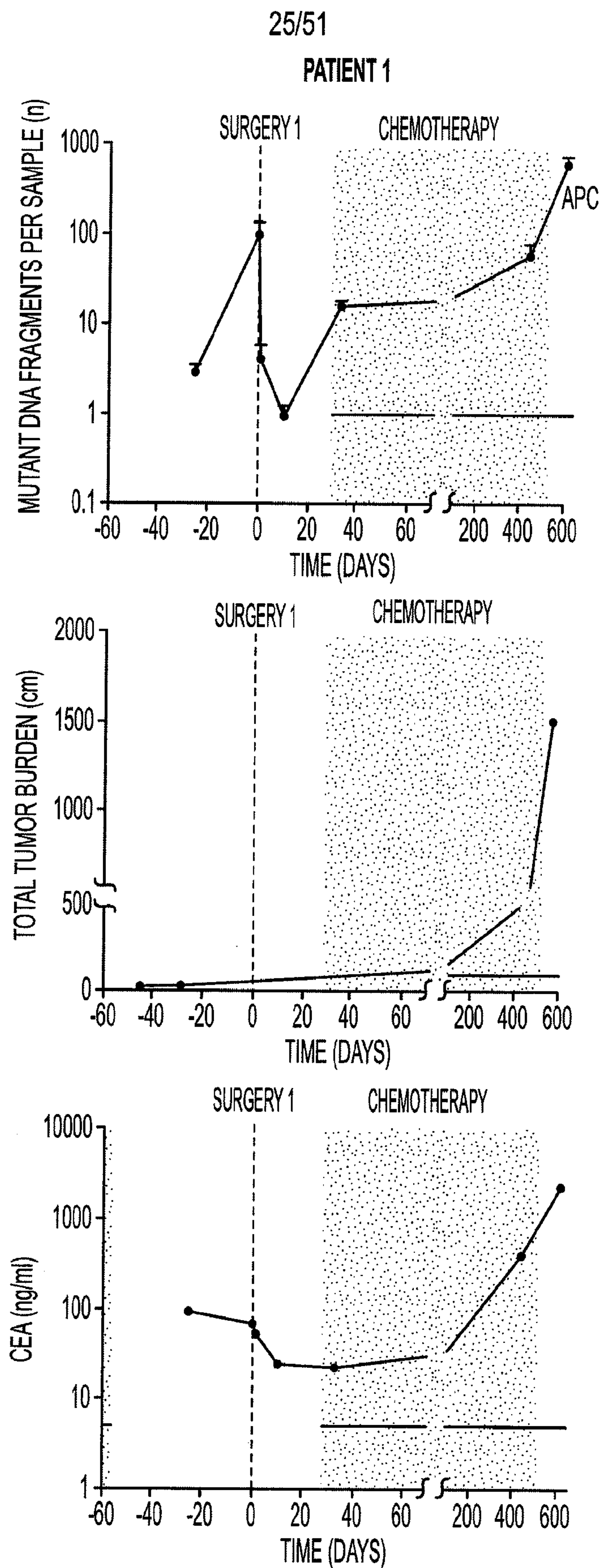


FIG. 16A-1

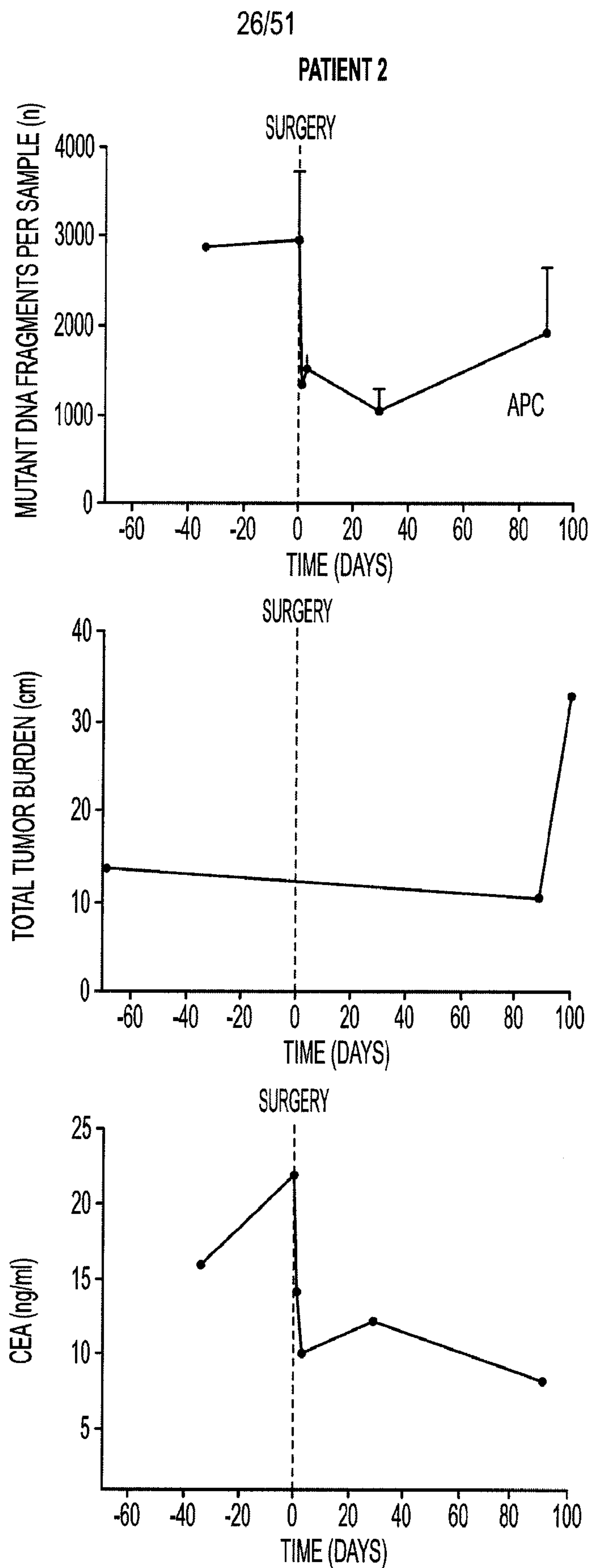


FIG. 16A-2

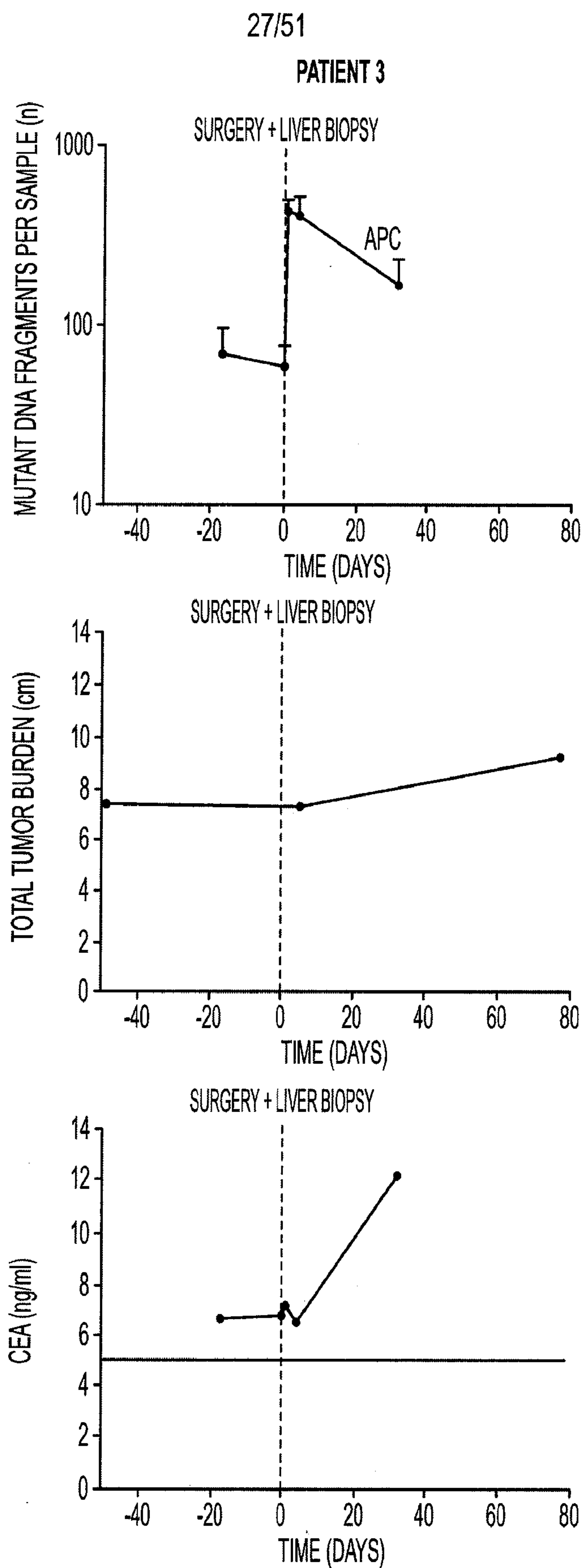


FIG. 16A-3

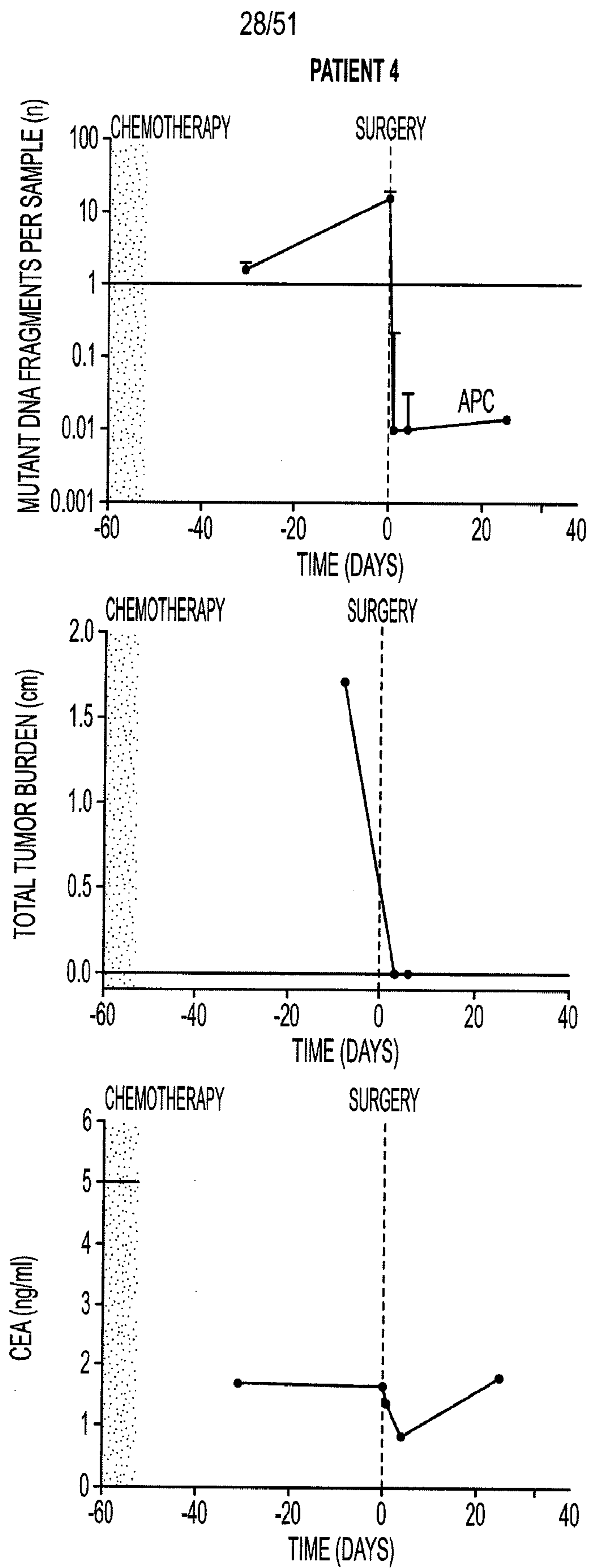


FIG. 16B-1

29/51

PATIENT 6

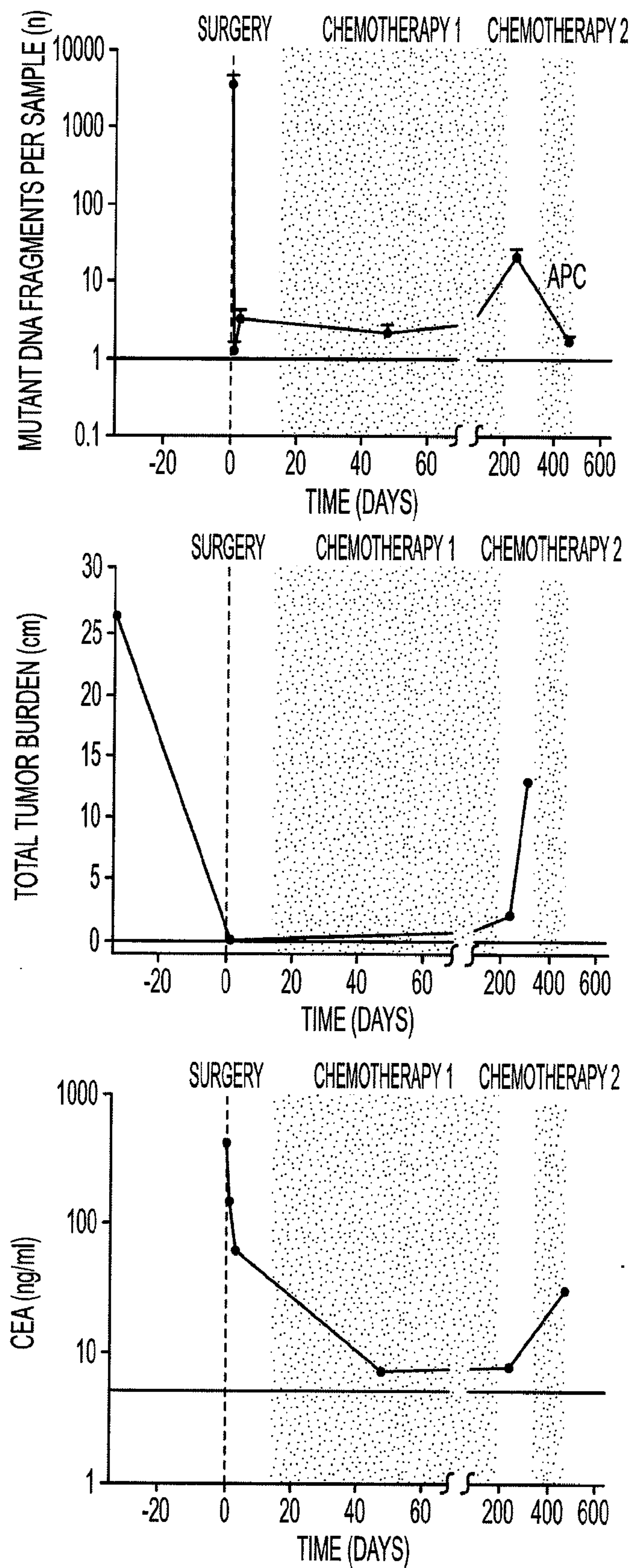
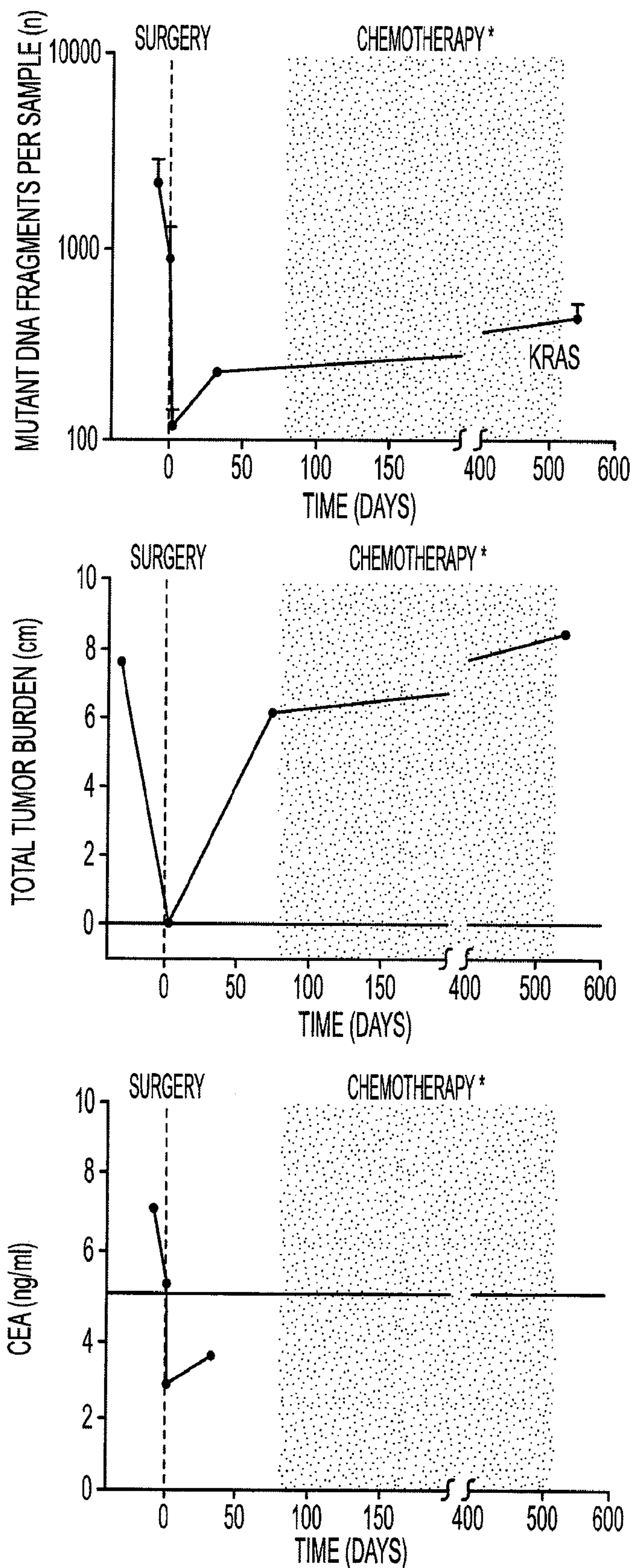


FIG. 16B-2

30/51

PATIENT 7



* NO EXACT DATES FOR START AND END OF CHEMOTHERAPY RECORDED

FIG. 16B-3

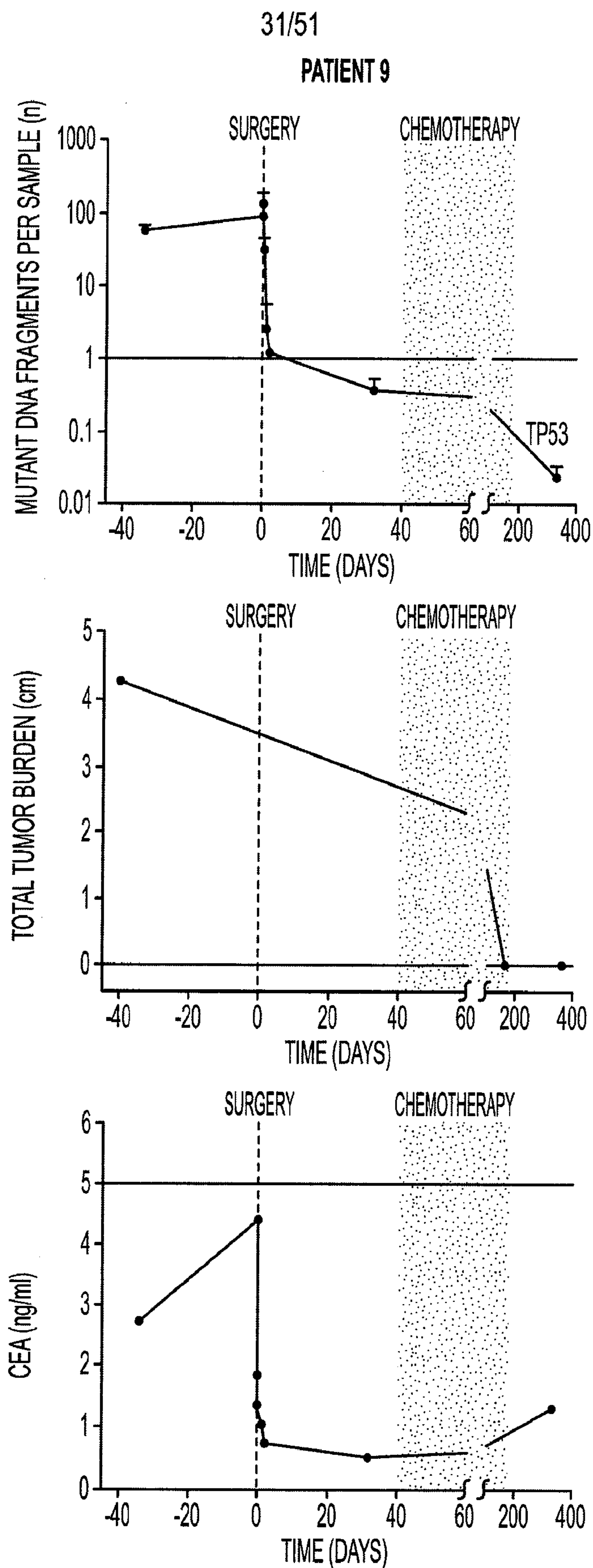


FIG. 16C-1

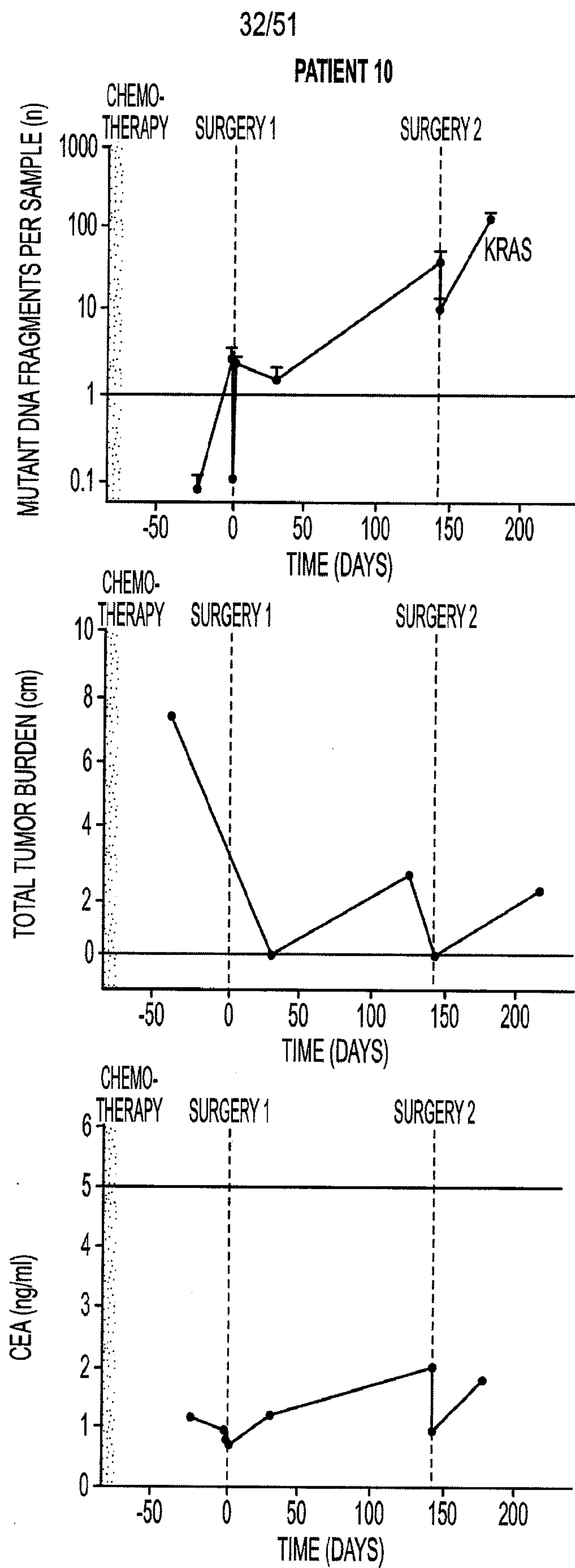


FIG. 16C-2

33/51

PATIENT 12

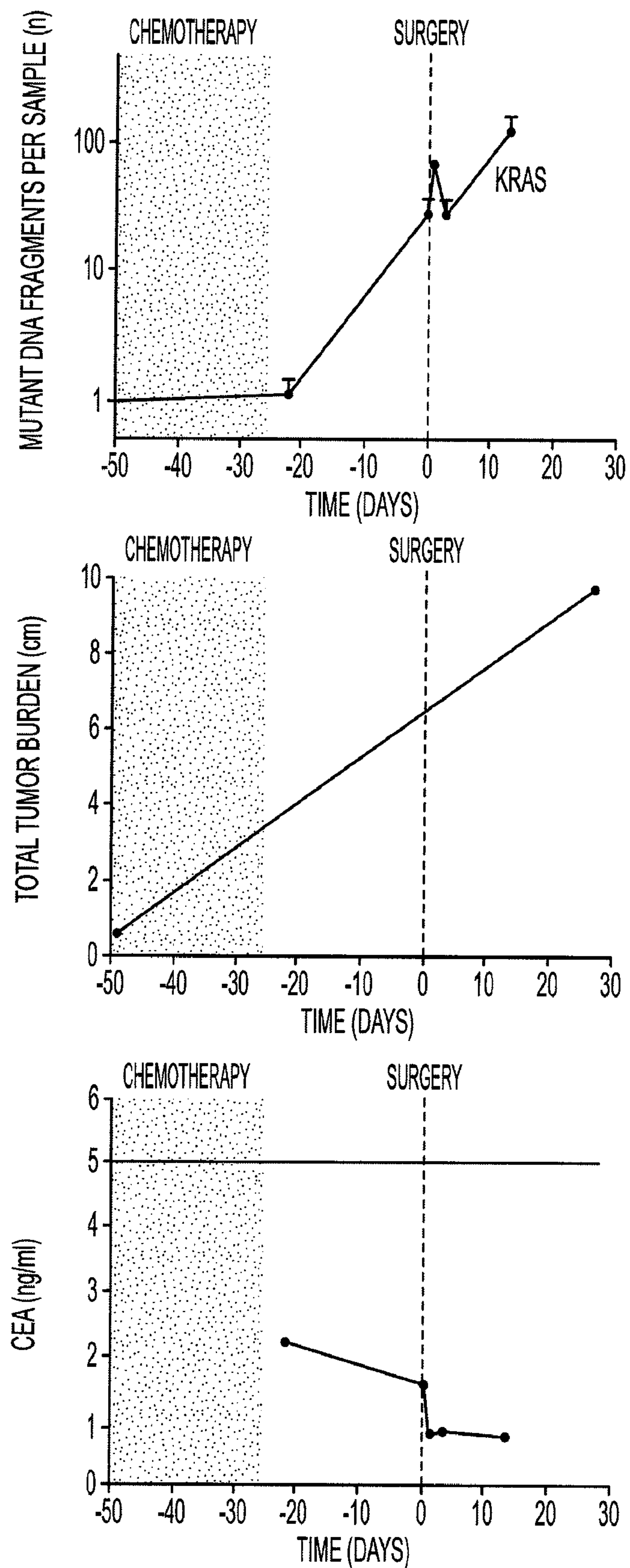


FIG. 16C-3

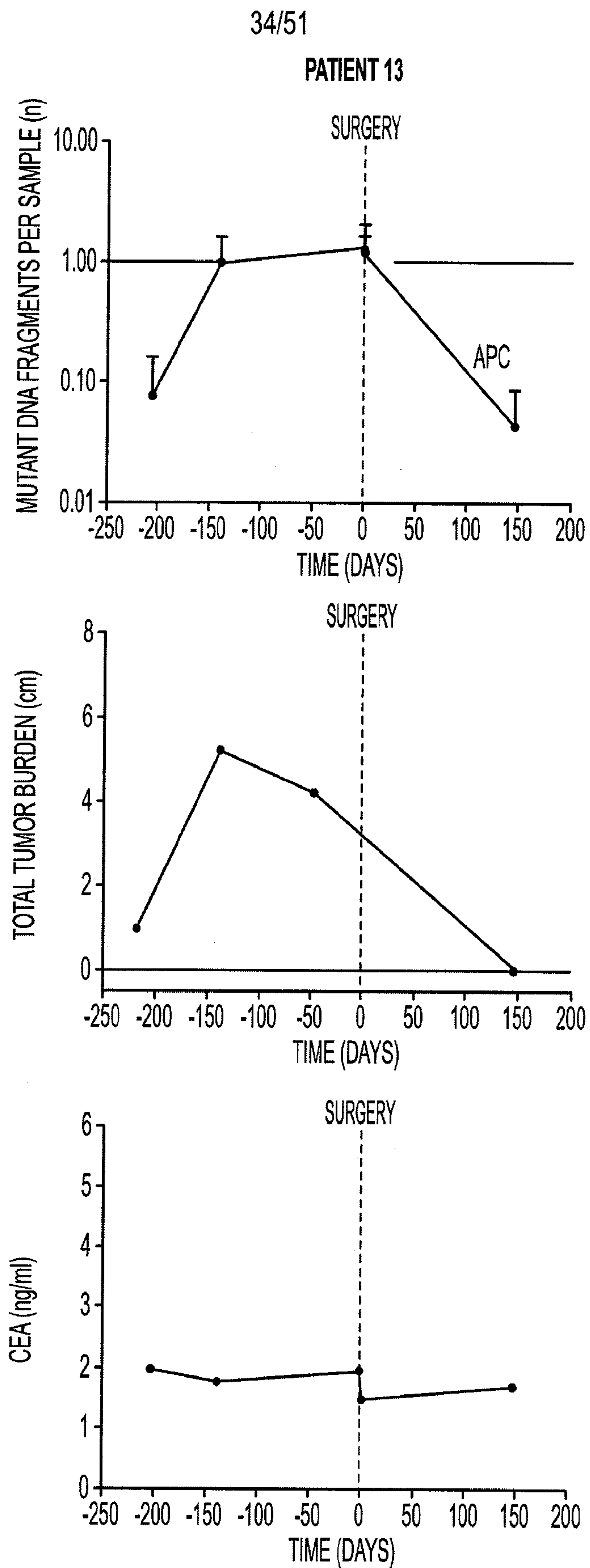


FIG. 16D-1

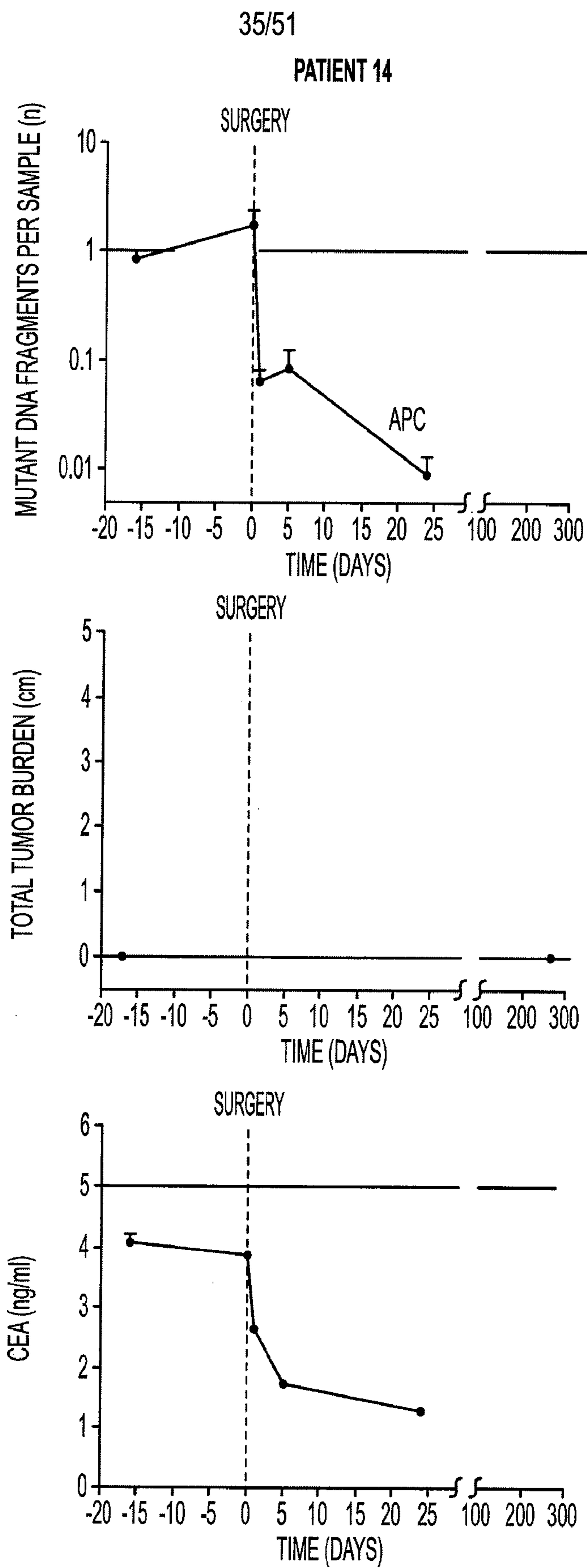


FIG. 16D-2

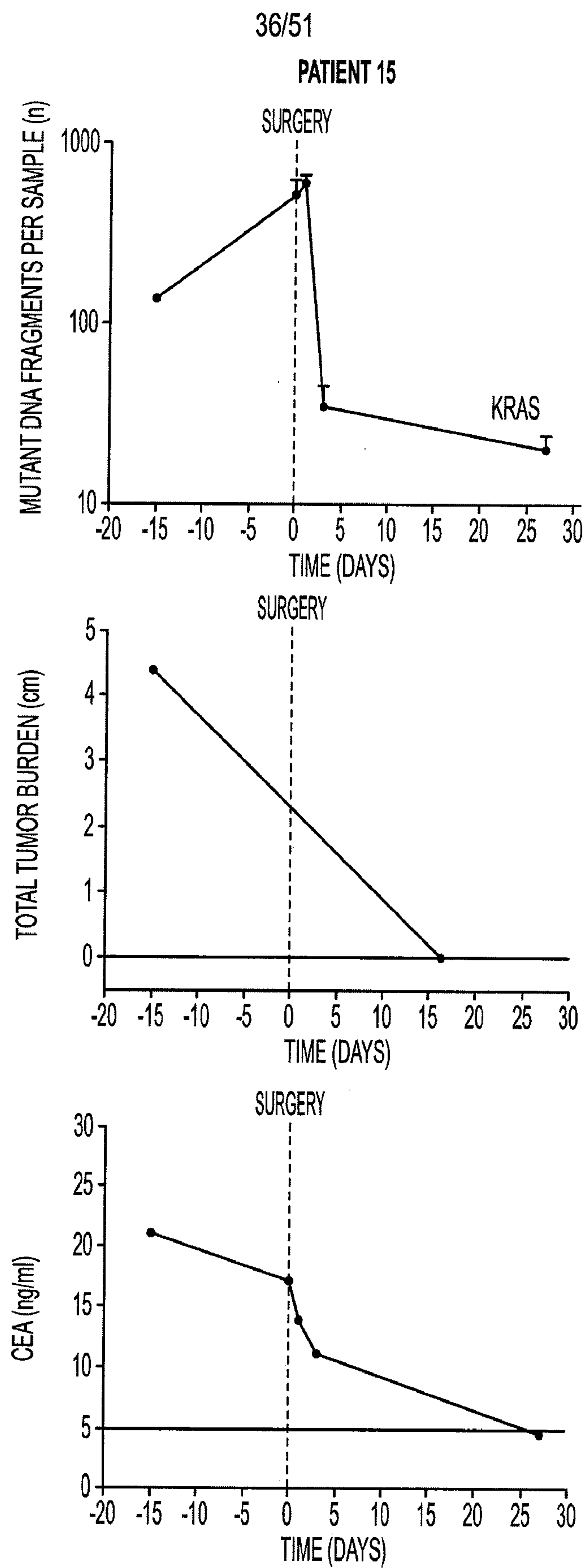


FIG. 16D-3

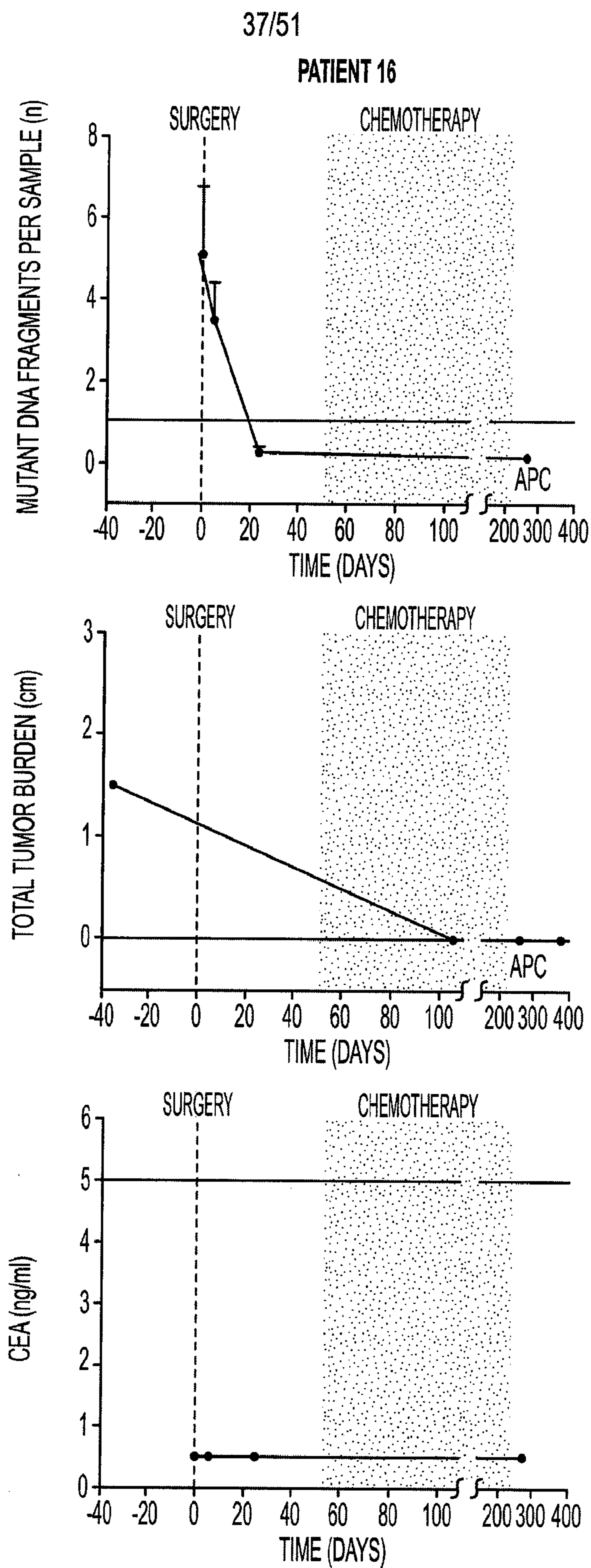


FIG. 16E-1

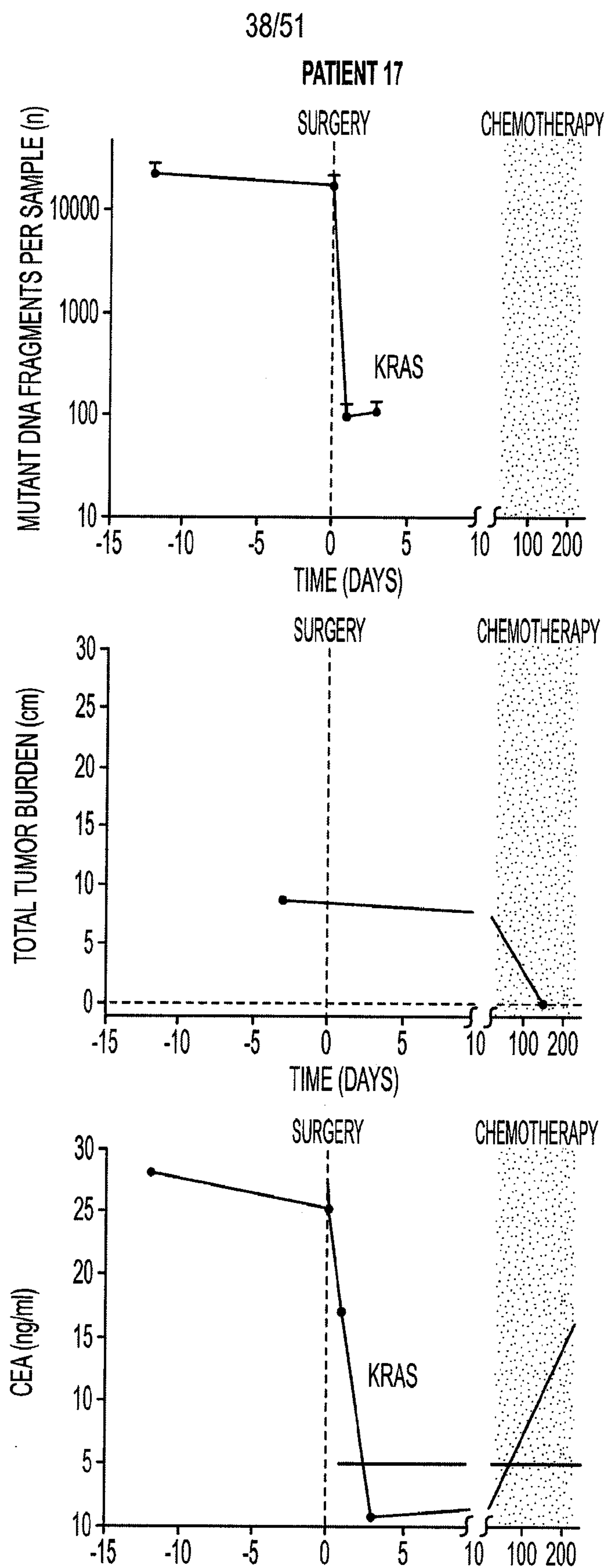


FIG. 16E-2

39/51

PATIENT 18

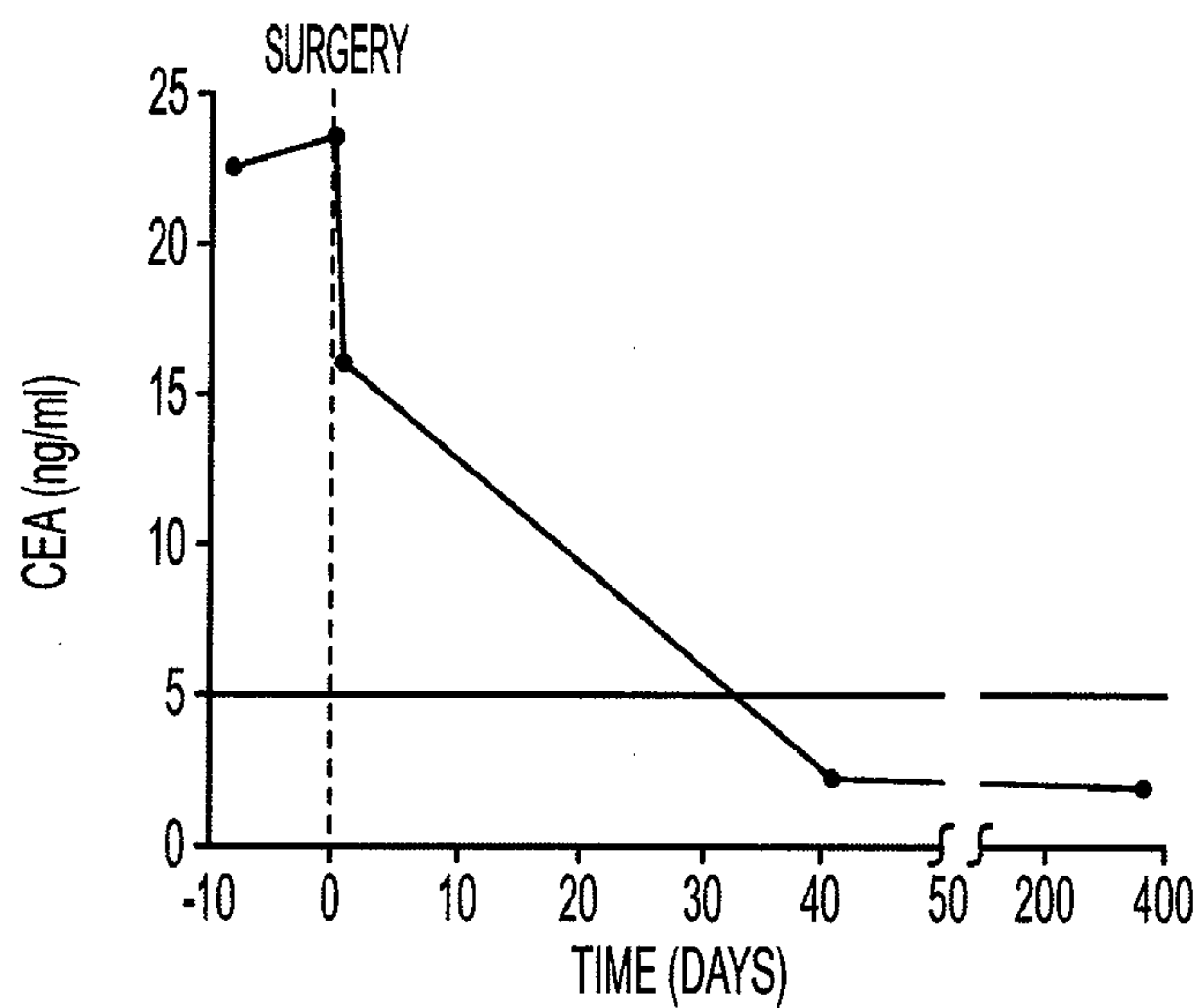
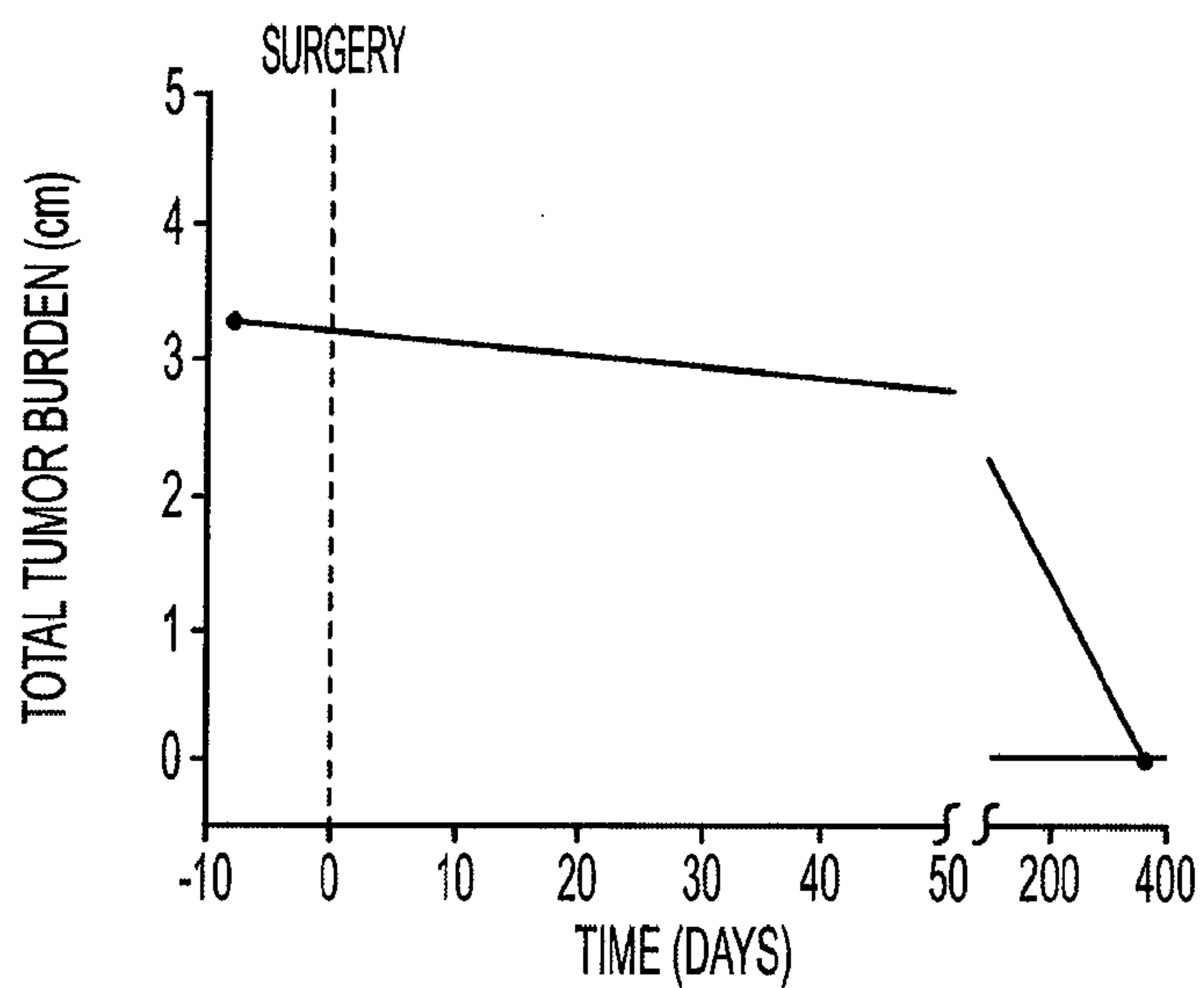
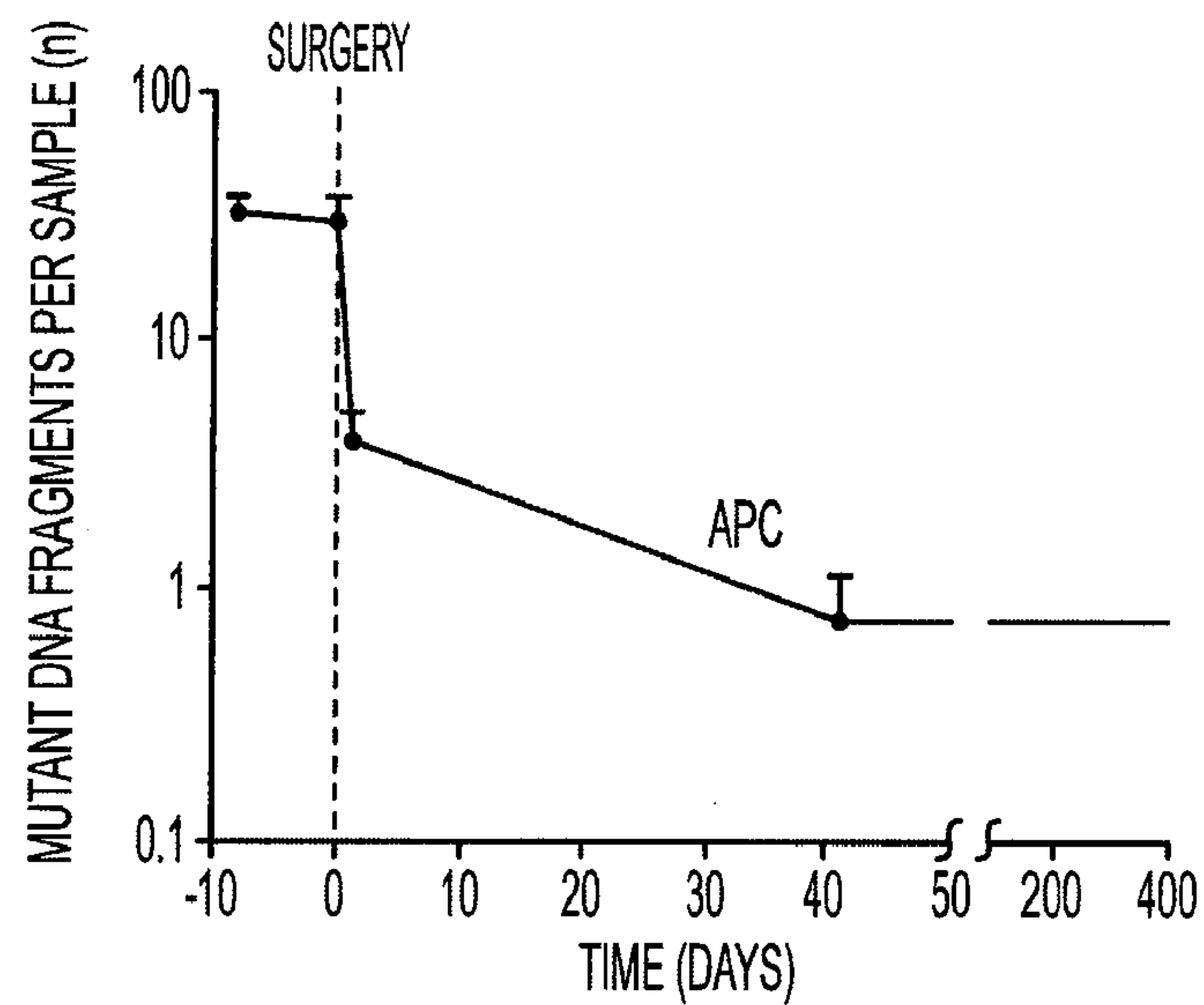


FIG. 16E-3

40/51

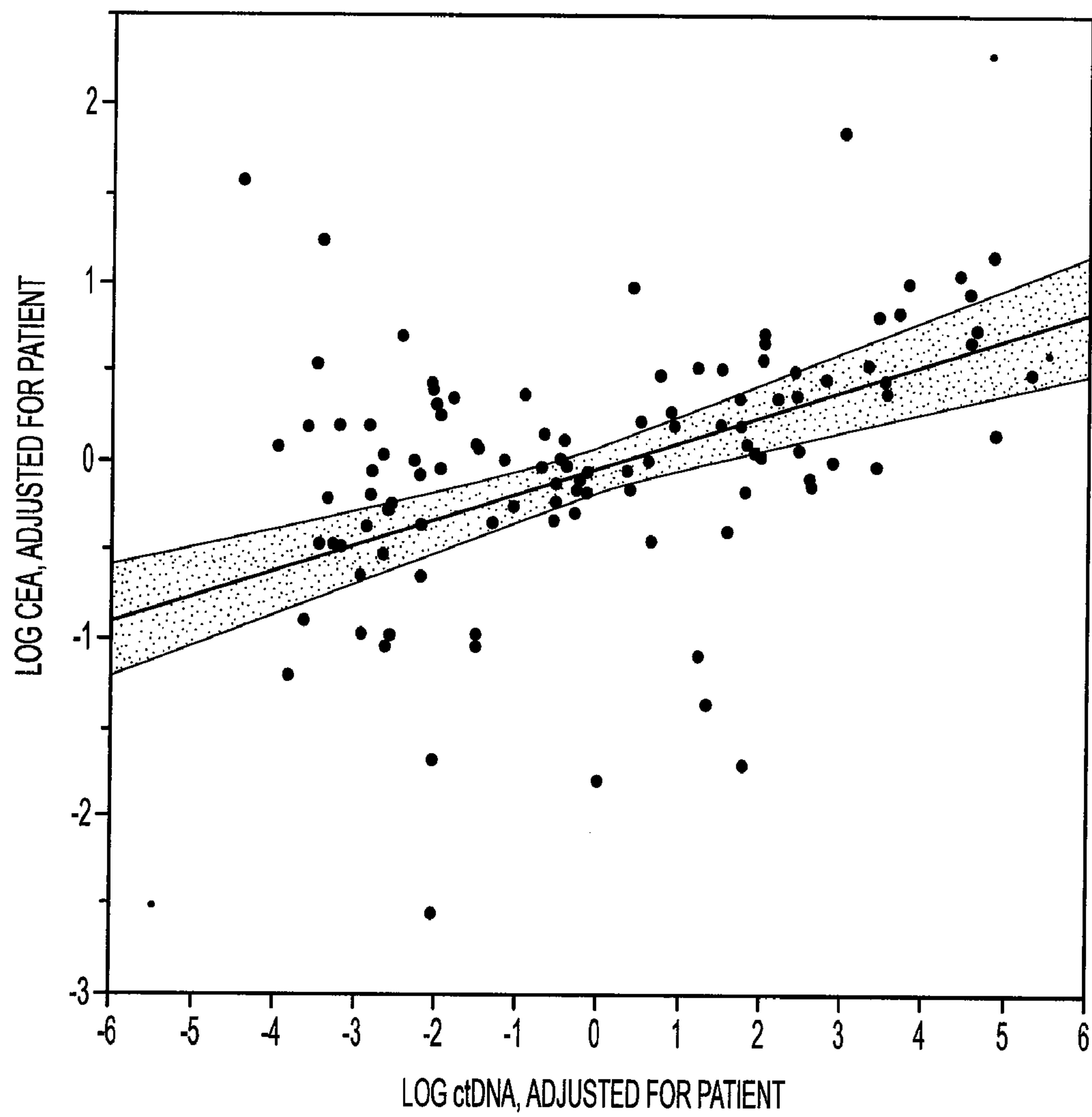
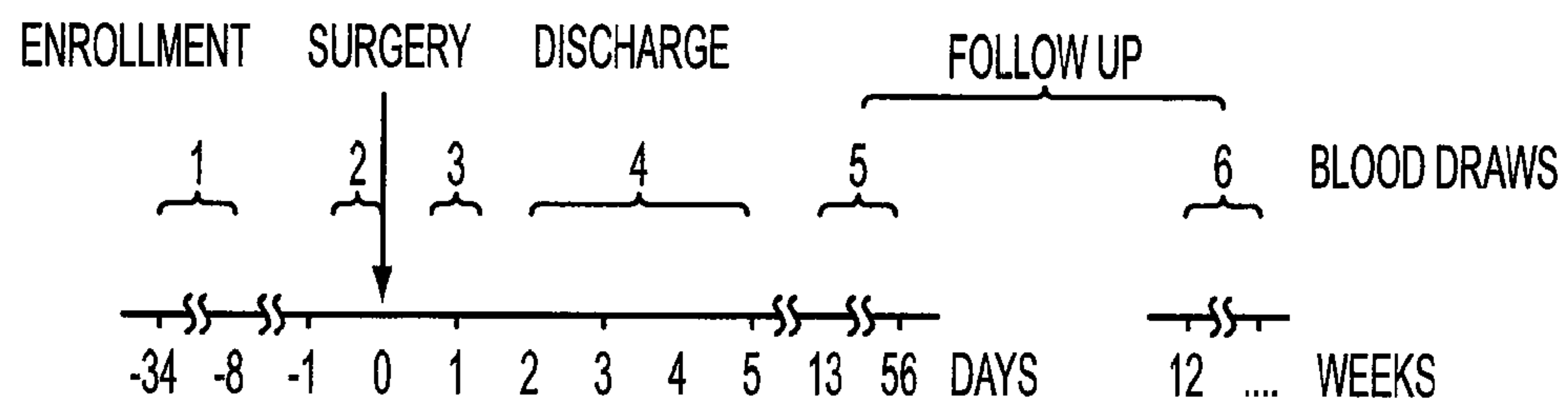


FIG. 17

41/51



PLASMA COLLECTION TIME-LINE

FIG. 18

42/51

PATIENT NO.	GENE	MUTATION (CODON)	PLASMA ANALYZED
1	APC	4461del t (1487)	YES
	KRAS	G38A (13)	YES
	TP53	C817T (273)	NO
2	APC	C4031A (1344)	YES
3	APC	C4348T (1450)	YES
	KRAS	G35C (12)	YES
	TP53	G733A (245)	NO
4	APC	4465-4468del TTAC (1489)	YES
	KRAS	G38A (13)	YES
	TP53	G730A (244)	NO
5	PIK3CA	G1624A (542)	YES
	TP53	C844T (282)	YES
6	APC	G4189T (1397)	YES
	KRAS	G35A (12)	YES
	TP53	G743A (248)	NO
7	KRAS	G35T (12)	YES
8	PIK3CA	G1624A (542)	YES
	TP53	G818A (273)	YES
9	TP53	C535T (179)	YES
10	APC	C4067A (1356)	YES
	KRAS	G35A (12)	YES
11	TP53	C817T (273)	YES
12	PIK3CA	A3140G (1047)	NO
	KRAS	G38A (13)	YES
13	APC	G3862T (1288)	YES
14	APC	3877-3889del ACACAGGAAGCAG	YES
15	KRAS	G35T (12)	YES
16	APC	3905del T (1302)	YES
	TP53	C844T (282)	YES
17	APC	C2626T (876)	YES
	KRAS	G35A (12)	YES
18	APC	C4012T (1338)	YES

FIG. 19

43/51

AMPLICON NO	GENE	EXON	PCR	FORWARD PRIMER 5'-3'	REVERSE PRIMER 5'-3'
1	APC	5	1ST 2ND	tgaagcaaggcaaatacagagt M13-caaatcagagttgcgatgga	tcgctgtttatcactagaaacaa TAG1-tcgctgtttatcactagaaacaa
2	APC	6	1ST 2ND	acataactaattaggttctgttttattt M13-acataactaattaggttctgttttattt	cctctgcttctgttgcctgg TAG1-tgctgggactgtaaagctg
3	APC	15	1ST 2ND	ggcaactaccatccagcaac M13-tccagcaacagaaaaatccag	atctgggctgcagtggtg TAG1-atctgggctgcagtggtg
4	APC	15	1ST 2ND	tggttctccatacaggcacg M13-gtcacggggagccaatg	tggcttacatttgattaattccat TAG1-tggcttacatttgattaattccat
5	APC	15	1ST 2ND	tccaatatgttttcaagatgtagttc M13-tccaatatgttttcaagatgtagttc	cagaatctgcttctgtgtcg TAG1-tctgcttctgtgtcgtctg
6	APC	15	1ST 2ND	ctgaagatgaaataggatgtaacagac TAG1-ctgaagatgaaataggatgtaacagac	cttcagctgacctagtccaatc M13-cttcagctgacctagtccaatc
7	APC	15	1ST 2ND	cagattctgctaataccctgcaa TAG1-cagattctgctaataccctgcaa	agggctgtgtgacactgctg M13-actgtctggaacttcgctcac
8	APC	15	1ST 2ND	ttggaactaggtcagctgaaga TAG1-ttggaactaggtcagctSaaga	gaagataaactagaacctgcagtc M13-gcagctcgtctggatttggtt
9	APC	15	1ST 2ND	gatcctgtgagcgaagtcc M13-agcgaagtccagcagtgtc	tgcttggtgattctgaag TAG1-tgcttggtgattctgaag
10	APC	15	1ST 2ND	cagcagactgcagggttctag M13-cagcagactgcagggttctag	gtctgagcaccactttggag TAG1-ccactttggaggagatttc
11	APC	15	1ST 2ND	tcttcaggagcgaaatctcc TAG1-tcttcaggagcgaaatctcc	gctaaacatgagtgggtctc M13-atgagtgggtctcctgaac
12	APC	15	1ST 2ND	ccaaaagtgggtctcagaca M13-gctcagacacccaaaagtcc	caaaaactatcaagtgaactgacagaag TAG1-caaaaactatcaagtgaactgacagaag
13	APC	15	1ST 2ND	gacccactcatgttttagcag TAG1-gacccactcatgttttagcag	tgccacttaccattccactg M13-cattccactgcatgggtcac



TO FIG. 20B

FIG. 20A

44/51

FROM FIG. 20A



14	APC	15	1ST	agtcgttcgattgccagctc	catggtttgtccagggctatc
			2ND	M13-cgattgccagctccgttc	TAG1-catggtttgtccagggctatc
15	APC	15	1ST	ccatgcagtggaatggtlaag	ggiggagggttttactctgc
			2ND	M13-tggcattataagccccagtg	TAG1-ggtggagggttttactctgc
16	APC	15	1ST	gccctggacaaacctgc	agcagtaggtgctttatcttagg
			2ND	M13-gacaaacctgccaccaag	TAG1-agcagtaggtgctttatcttagg
17	APC	15	1ST	cacctctcaaacagctcaa	gcagcatttactgcagcttg
			2ND	M13-tctcaaacagctcaaacca	TAG1-gcagcatttactgcagcttg
18	APC	15	1ST	gcagtaaatgctgcagtcagag	tcaatatcatcatctgaatcatc
			2ND	M13-cagtcagagggtccaggtt	TAG1-cactcaggctggatgaacaa
19	APC	15	1ST	gcctaaagaatcaaatgaaaacc	atcatcatctgaatcatctaagggtc
			2ND	M13-caaatgaaaaccaagagaaagagg	TAG1-atcatcatctgaatcatctaagggtc
20	PIK3CA	9	1ST	gcaatttctacacgagatcctct	tccattttagcacttacctgtgac
			2ND	TAG1-gcaatttctacacgagatcctct	M13-cttacctgtgactccatagaaaatc
21	PIK3CA	20	1ST	ctgagcaagaggctttggag	tggttggaagatccaatcca
			2ND	TAG1-ctgagcaagaggctttggag	M13-tccaatccattttgtgtcc
22	TP53	5	1ST	cggcatggccatctacaag	ctcaccatcgctatctgagc
			2ND	TAG1-tggccatctacaagcagtca	M13-ctcaccatcgctatctgagc
23	TP53	6	1ST	taggtctggccccctctc	cagttgcaaaccagacctca
			2ND	M13-gccccctctcagcatcttat	TAG1-cagttgcaaaccagacctca
24	TP53	7	1ST	aggttggctctgactgtacca	tctccagtgtgatgatggtg
			2ND	TAG1-aggttggctctgactgtacca	M13-agtgtgatgatggtgaggatg
25	TP53	8	1ST	atctactgggacggaacagc	cccttcttgcggagattc
			2ND	M13-atctactgggacggaacagc	TAG1-cttgcggagattctcttct
26	KRAS	1	1ST	ttattataaggcctgctgaaaatg	tagctgtatcgtcaaggcactc
			2ND	TAG1-ttattataaggcctgctgaaaatg	M13-cgtcaaggcactcttgcc

TAG 1:5'-tcccgcgaaattaatacgac
M13:5'-gtaaaacgacggccagt

FIG. 20B

45/51

AMPLICON NO.	GENE	EXON	PATIENT NO.	PCR	SIZE (bp)	FORWARD PRIMER 5'-3'	REVERSE SEQUENCE 5'-3'	PCR CONDITIONS	SIZE (bp) LINE 1 PCR
3	APC	15	17	1ST	99	TAG2-tggagagagaacgcggaattggt	TAG1-ctgcagtggtggagaictgcaaac	A	A
5	APC	15	13	1ST	116	gtgtagaagatactccaatatgttttcaagatgtagttc	gtattagcagaatctgtcttctgtgtcg	B	97 & 127
				2ND	108	TAG2-gtgtagaagatactccaatatgttttcaagatgtagttc	TAG1-cagaatctgtcttctgtgtgtgtgt	E	
6	APC	15	14, 16	1ST	120	TAG1-ctgaagatgaaataggatgtaatcagac	TAG2-cacaggatctcagctgacctagtccaac	A	127
9	APC	15	18	1ST	95	TAG2-tgtgagcgaaagtccagcagtgtc	TAG1-ctttgtgcttgctgattctgaag	A	97
10	APC	15	2, 10	1ST	110	aaatccagcagactgcagggttctag	ggtgtctgagcaccacttttgag	A	97 & 127
				2ND	102	TAG2-aaatccagcagactgcagggttctag	TAG1-agcaccacttttgaggaggatitc	D	
13	APC	15	6	1ST	115	TAG1-caggagacccactcatgttttagcag	TAG2-cacttaeccactcctgcatgtgtcac	A	97 & 127
16	APC	15	3	1ST	98	TAG2-gacaaaccatgccaccaag	TAG1-agcagtaggtgcttatttttagg	C	97
18	APC	15	1, 4	1ST	111	atgtctgcagttcagagggtccag	tcagagcactcaggctggatgaac	A	97 & 127
				2ND	106	TAG2-gcagttcagagggtccaggttcttc	TAG1-tcagagcactcaggctggatgaac	D	
20	PIK3CA	9	5, 8	1ST	101	gctcaaagcattttacacgagatcctct	cagagaatctccattttagcacttacctgtgac	A	97
				2ND	90	TAG1-gctcaaagcattttacacgagatcctct	TAG2-catttttagcacttacctgtgactccatagaaaatc	D	
22	TP53	5	9	1ST	84	TAG1-tctacaagcagtcacagcacatgacg	TAG2-gctgctcaccatcgctatctgagc	A	79 & 97
25	TP53	8	5, 8, 11 16	1ST	95	TAG2-tggtaatclactgggacggaacagctt	TAG1-ctttcttgggagattctcttctctg	A	97
26	KRAS	1	1, 3, 4, 6, 7, 10, 12 15, 17	1ST	96	TAG2-tgactgaataaaactgtgtgtagttg	TAG1-catattctgtccacaaaatcatic	C	97

TAG1: 5'-tcccgcgaaattaatcagac
TAG2: 5'-gctggagctctgcagcta

- A. 98°C, 30 s; 37x (98°C, 10 s, 71°C, 10 s)
B. 98°C, 30 s; 35x (98°C, 10 s, 69°C, 72°C, 10 s)
C. 98°C, 30 s; 3x (98°C, 10 s, 70°C, 72°C, 10 s), 3x (98°C, 10 s, 67°C, 10 s, 72°C, 10 s),
3x (98°C, 10 s, 64°C, 72°C, 10 s), 28x (98°C, 10 s, 61°C, 10 s, 72°C, 10 s),
D. 98°C, 30 s; 4x (98°C, 10 s, 71°C, 10 s)
E. 98°C, 30 s; 4x (98°C, 10 s, 69°C, 10 s, 72°C, 10 s)

FIG. 21

46/51

GENE	MUTATION	PATIENT NO.	AMPLICON NO.	PROBE	PROBE SEQUENCE 5'-3'
APC	C2626T	17	3	UNIVERSAL WILD-TYPE MUTANT	ROX-agcaacagaaaatccagga Cy3-cttcaaagcgaggtttg Cy5-cttcaaagtgaggtttg
APC	G3862T	13	5	UNIVERSAL WILD-TYPE MUTANT	ROX-gtagttcattatcatcttt Cy3-atgaaataggatgtaatc Cy5-atgaaatatgatgtaatc
APC	3877 - 3889 del ACACA GGAAGCAG	14	6	UNIVERSAL WILD-TYPE MUTANT	ROX-tagttccaatcttttctttt Cy3-atctgcttcctgtgtcg Cy5-tagcagaatcgctgat
APC	3905delT	16	6	UNIVERSAL WILD-TYPE MUTANT	ROX-tagttccaatcttttctttt Cy3-ctattgcagggtatta Cy5-ctattgcgggtattag
APC	C4012T	18	9	UNIVERSAL WILD-TYPE MUTANT	ROX-cacagcacctagaaccaa Cy3-agcagactgcagggtt Cy5-agcagactgtagggtt
APC	C4031A	2	10	UNIVERSAL WILD-TYPE MUTANT	ROX-ttcttcaggagcgaaatct Cy3-tttatcttcagaatcagc Cy5-tttatcttaagaatcagc
APC	C4067A	10	10	UNIVERSAL WILD-TYPE MUTANT	ROX-tagtttatcttcagaatca Cy3-attttcttcaggagcga Cy5-attttcttaaggagcga
APC	G4189T	6	13	UNIVERSAL WILD-TYPE MUTANT	ROX-aactgacagaagtacatct Cy3-acgactctcaaaactat Cy5-acgactctaaaaactat
APC	C4348T	3	16	UNIVERSAL WILD-TYPE MUTANT	ROX-cagaagtaaaacacctcca Cy3-aaaccaagcgagaagta Cy5-aaaccaagtgagaagta
APC	4461delT	1	18	UNIVERSAL WILD-TYPE MUTANT	ROX-tcagagggtccagggtctt Cy3-gctgatactttattaca Cy5-gctgatacttattacat



TO FIG. 22B

FIG. 22A

47/51

FROM FIG. 22A



APC	4465 - 4468 del TTAC	4	18	UNIVERSAL WILD-TYPE MUTANT	ROX-tcagaggggtccaggttctt Cy3-tactttattacattttgc Cy5-gatactttaatttgcca
PIK3CA	G1624A	5.8	20	UNIVERSAL WILD-TYPE MUTANT	ROX-acctgtgactccatagaaa Cy3-agtgatttcagagagag Cy5-agtgattttagagagag
TP53	C535T	9	22	UNIVERSAL WILD-TYPE MUTANT	ROX-cctcacaacctccgtcatg Cy3-gcgctcatggtgggggc Cy5-gcgctcatagtgggggc
TP53	C817T	11	25	UNIVERSAL WILD-TYPE MUTANT	ROX-cctgggagagaccggcgca Cy3-tgaggtgcgtgtttgtg Cy5-tgaggtgtgtttgtg
TP53	G818A	8	25	UNIVERSAL WILD-TYPE MUTANT	ROX-cctgggagagaccggcgca Cy3-tgaggtgcgtgtttgtg Cy5-tgaggtgcatgtttgtg
TP53	C844T	5, 16	25	UNIVERSAL WILD-TYPE MUTANT	ROX-tgaggtgcgtgtttgtgcc Cy3-tgagagaccggcgccaca Cy5-tgagagactggcgccaca
KRAS	G35A	6, 10, 17	26	UNIVERSAL WILD-TYPE MUTANT	ROX-tgacgatacagctaattca Cy3-ggagctggtggcgta Cy5-ggagctgatggcgta
KRAS	G35C	3	26	UNIVERSAL WILD-TYPE MUTANT	ROX-tgacgatacagctaattca Cy3-ggagctggtggcgta Cy5-ggagctgctggcgta
KRAS	G35T	7, 15	26	UNIVERSAL WILD-TYPE MUTANT	ROX-tgacgatacagctaattca Cy3-ggagctggtggcgta Cy5-ggagctgttggcgta
KRAS	G38A	1, 4, 12	26	UNIVERSAL WILD-TYPE MUTANT	ROX-tgacgatacagctaattca Cy3-tgctggtggcgtaggc Cy5-tgctggtgacgtaggc

FIG. 22B

48/51

CHARACTERISTICS OF PATIENTS	
CHARACTERISTIC	VALUE (N=18)
AGE - YEARS	
MEAN	59.8
RANGE	35-82
SEX	
MALE	8 (44%)
FEMALE	10 (56%)
STAGE	
IV	16 (88%)
III	1 (6%)
II	1 (6%)
DIFFERENTIATION	
WELL	3 (17%)
MODERATE	11 (61%)
POOR	2 (11%)
UNSPECIFIED	2 (11%)
LOCATION OF METASTASES	
LIVER	15
LUNG	2
OMENTUM OR PERITONEAL	2
NUMBER OF SURGERIES	
1	15
2	2
3	1
PREOPERATIVE CEA (ng/ml)	
MEAN	42
RANGE	0.5-2,250
% ABOVE NORMAL RANGE (>5ng/ml)	55%

FIG. 23

49/51

PATIENT NO.	STAGE	SURGERY NO.	SURGICAL PROCEDURE
1	IV	1	HEPATIC METASTECTOMY
2	IV	1	HEPATIC METASTECTOMY (FIRST-STAGE)
3	IV	1	RIGHT HEMICOLECTOMY WITH HEPATIC METASTECTOMY
4	IV	1	HEPATIC METASTECTOMY WITH RFA
5	IV	1	HEPATIC METASTECTOMY
6	IV	1	PULMONARY AND HEPATIC METASTECTOMY
7	IV	1	HEPATIC METASTECTOMY WITH RFA
8	IV	1	SIGMOID COLECTOMY WITH HEPATIC METASTECTOMY (FIRST STAGE)
		2	RIGHT HEPATECTOMY
9	IV	1	RIGHT HEPATECTOMY
10	IV	1	RIGHT HEMICOLECTOMY WITH HEPATIC METASTECTOMY
		2	HEPATIC METASTECTOMY WITH RFA
11	IV	1	SIGMOID COLECTOMY WITH HEPATIC METASTECTOMY WITH RFA
		2	HEPATIC METASTECTOMY
		3	RFA TO HEPATIC METASTASES
12	IV	1	HEPATIC METASTECTOMY WITH RFA
13	IV	1	HEPATIC METASTECTOMY WITH PARTIAL GASTRECTOMY AND OMENTECTOMY
14	II	1	SIGMOID COLECTOMY (T3N0M0)
15	IV	1	RIGHT HEPATECTOMY
16	III	1	SIGMOID COLECTOMY (T3N1M0)
17	IV	1	RIGHT HEPATECTOMY
18	IV	1	HEPATIC METASTECTOMY

TO
FIG. 24B

*MEANS \pm 1 S.D. ARE SHOWN
 NEG, ctDNA UNDETECTABLE
 -, SAMPLE NOT DONE

FIG. 24A

50/51

ctDNA LEVEL (ctDNA MOLECULES/2ml)*

RESECTION	GENE	POST-OPERATIVE			
		PRE-SURGERY	POST-OPERATIVE	POST-OPERATIVE	FOLLOW-UP
		DAY 0	DAY 1	DAY 2-10	DAY 13-56
COMPLETE	APC	99 ± 38	4.0 ± 2.0	0.9 ± 0.3	16 ± 2.3
	KRAS	79 ± 35	2.6 ± 1.3	NEG	5.6 ± 0.9
INCOMPLETE	APC	2951 ± 773	1336 ± 408	1508 ± 156	1049 ± 254
INCOMPLETE	APC	58 ± 18	421 ± 85	404 ± 121	166 ± 63
	KRAS	46 ± 15	485 ± 98	393 ± 118	136 ± 51
COMPLETE	APC	16 ± 4	NEG	NEG	NEG
	KRAS	15 ± 4	NEG	NEG	NEG
COMPLETE	PIK3CA	213 ± 90	4.9 ± 2.1	7.7 ± 2.3	9.8 ± 3.8
	TP53	275 ± 116	4.5 ± 2.0	2.6 ± 0.8	5.7 ± 1.7
COMPLETE	APC	3650 ± 1313	1.3 ± 0.4	3.3 ± 1.3	2.2 ± 0.6
	KRAS	2375 ± 917	1.2 ± 0.5	4.4 ± 1.7	1.2 ± 0.4
COMPLETE	KRAS	856 ± 394	117 ± 24	-	220 ± 9
INCOMPLETE	PIK3CA	2575 ± 1027	1370 ± 551	6459 ± 2305	1420 ± 408
	TP53	2352 ± 937	802 ± 248	4171 ± 1484	944 ± 258
COMPLETE	PIK3CA	644 ± 281	26.5 ± 7.9	3.7 ± 1.2	0.9 ± 0.1
	TP53	389 ± 169	5.9 ± 1.7	NEG	NEG
COMPLETE	TP53	91 ± 33	2.6 ± 3.3	1.2 ± 1.3	NEG
COMPLETE	APC	6.0 ± 1.6	NEG	NEG	NEG
	KRAS	2.8 ± 1.0	NEG	2.4 ± 0.5	1.5 ± 0.7
COMPLETE	APC	128 ± 34	37 ± 14	-	316 ± 64
	KRAS	42 ± 13	11 ± 5	-	131 ± 32
INCOMPLETE	TP53	14 ± 3	59 ± 22	-	295 ± 71
COMPLETE	TP53	2713 ± 775	10 ± 5	14 ± 7	1.8 ± 0.4
COMPLETE	TP53	1267 ± 243	465 ± 8	-	39 ± 2
INCOMPLETE	KRAS	28 ± 8	67 ± 9	27 ± 9	120 ± 42
COMPLETE	APC	1.3 ± 0.3	1.2 ± 0.9	-	-
COMPLETE	APC	1.7 ± 0.7	NEG	NEG	NEG
COMPLETE	KRAS	519 ± 125	612 ± 60	35 ± 11	20 ± 4
COMPLETE	APC	5.1 ± 1.7	-	3.5 ± 0.9	NEG
	TP53	1.8 ± 0.6	-	1.6 ± 0.2	NEG
COMPLETE	APC	20144 ± 5504	58 ± 22	13 ± 3	-
	KRAS	17482 ± 4777	97 ± 36	108 ± 27	-
COMPLETE	APC	29 ± 8	3.91 ± 1.28	-	0.7 ± 0.4

FROM
FIG. 24ATO
FIG. 24C

FIG. 24B

51/51

CEA LEVEL (ng/ml)				
PRE-OPERATIVE DAY 0	POST-OPERATIVE DAY 1	POST-OPERATIVE DAYS 2-10	POST-OPERATIVE FOLLOW-UP DAY 13-56	DISEASE RECURRENCE
69	53	24	22	YES
22	14	10	12	YES
6.8	7.2	6.5	12	YES
1.6	1.4	0.8	1.8	NO
9.4	6.8	4.4	2.1	YES
422	149	61	7.2	YES
5.3	2.7	-	3.4	YES
4.5	2.4	1.6	3.8	YES
1.4	0.6	0.5	0.7	YES
4.4	1.1	0.7	0.5	NO
0.9	0.8	0.7	1.2	YES
2.0	0.9	-	1.8	YES
93	55	-	32	YES
74	43	22	2.8	YES
52	44	-	9.1	YES
1.6	0.8	0.8	0.7	YES
2.0	1.5	-	-	YES
3.9	2.7	1.7	1.3	NO
17	14	11	4.5	YES
<0.5	<0.5	<0.5	<0.5	NO
25	17	0.8	-	YES
24	16	-	2.2	NO

FROM
FIG. 24B

FIG. 24C