The Spontaneous Mutational Spectrum of Exon 2 and the High Melting Region of Exon 3 of the Human HPRT Gene

by

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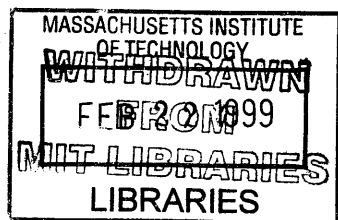
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Massachusetts Institute of Technology

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Abstract

As databases are growing for various disease-causing mutations, so does the suspicion that these mutations are not induced exogenously but may have arisen from endogenous mechanisms. In order to understand these mechanisms, we have set out to measure the spontaneous mutational spectrum of a sentinel gene to determine the predominant pathways leading to endogenous mutagenesis.

With recent technological advancements in mutational spectrometry, specifically the development of Constant Denaturant Capillary Electrophoresis (CDCE), coupled with high-fidelity PCR, the detection of one mutant sequence in one million wildtype sequences (10^{-6}) is possible. CDCE, which uses a constant temperature zone to separate mutant from non-mutant species (Khrapko, Hanekamp et al. 1994; Khrapko, Coller et al. 1996; Khrapko, Coller et al. 1997), was employed in an attempt to analyze the spontaneous point mutations in the human nuclear gene sequences based on position, kind, and frequency.

The spontaneous mutational spectra of exon 2 (bp 28-134) and the high melting region of exon 3 (bp 135-214) in the HPRT gene was examined in the TK-6 cell line. Human B-cells were grown in three six-liter cultures for approximately 60 consecutive cell doublings with daily dilution (back to 2.4 x 10^9 cells) to maintain exponential growth. These cells had a spontaneous mutation rate of approximately 2 x 10^{-7} HPRT mutations per cell doubling. Cells were grown in a large culture for many generations of exponential growth; mutants resistant to 6-thioguanine were then selected and directly amplified. The experiment was designed so even low frequency mutants ($\geq$ 0.5% 6TGR mutants) arising spontaneously could be measured with statistical precision. The results

At the mutant fraction level of 5×10^{-3} or greater, no common spontaneous hotspot were identified in the 71 basepairs analyzed of exon 2 from the replicate cultures after growing cells in culture for approximately 60 doublings. One small mutant was found in each of the two cultures both comprising less than 0.5% of the total 6TGR point mutations prior to enrichment. One spontaneous mutant was present in the high melting region of exon 3 in replicate cultures at a mutant fraction of 0.5% of the 6TGR point mutations.

This is the first time our laboratory has demonstrated the use of a CDCE assay introduced with a high melting clamp. This work shows other sequences that do not possess a naturally occurring low melting region followed by an adjacent high melting region can be analyzed by CDCE as previously demonstrated with DGGE and CDGE. The HPRT mutational database and the previously determined spontaneous spectrum in a mismatch repair deficient mutator cell line appear to have large hotspots in the regions that were analyzed, however no large hotspots (>1% of all 6TGR mutants) were found in the mismatch repair proficient cell line.

Thesis Supervisor: William G. Thilly
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List of Abbreviations

CDCE   Constant Denaturant Capillary Electrophoresis

PCR   Polymerase Chain Reaction

bp   Basepair

HPRT   Hypoxanthine-guanine phosphoribosyltransferase

6TG   6-Thioguanine

6TGR   6-Thioguanine Resistant

MMR   Mismatch repair

BER   Base excision repair

NER   Nucleotide excision repair

5mC   5-methylcytosine

HNPCC   Hereditary non-polyposis colorectal cancer

T_M Melting temperature

Pol-α Human DNA polymerase alpha

Pol-β Human DNA polymerase beta

Pol-γ Human DNA polymerase gamma

MNNG   N-methyl-N'-nitro-N-nitrosoguanidine

dNTPs   2'-deoxynucleoside-5'-triphosphates

Pfu   Pyrococcus furiosus

DGGE   Denaturant Gradient Gel Electrophoresis

BPDE   Benzo(a)pyrene diol epoxide

1. Introduction and Significance

The study of a 'background' mutational spectrum provides insight into the process of somatic mutation derivation that may be applicable to genes critical for cancer progression and human disease (Burkhart-Schultz, Thompson et al. 1996). Background or spontaneous mutations are those mutations that arise in cells without the induction of a known exogenous agent. DNA modifications occurring from the interaction with endogenous alkylating agents (Archer 1989), or oxidative species (Oller and Thilly 1992; Rossman and Goncharova 1998), erroneous DNA replication (Loeb, Liu et al. 1983), depurination/depyrimidation and deamination represent several pathways that may allow spontaneous mutations to arise in the nucleus if not corrected by DNA repair (Kunkel, Schaaper et al. 1983; Cooper and Krawczak 1989; Shen, Rideout et al. 1992). Additionally, enzymes involved in maintaining the genome, DNA replication, cell cycle checkpoints, or the failure of such enzymes can lead to mutations (Kaufmann and Paules 1996). Spontaneous mutations from any of these pathways are ultimately formed if they escape correction mechanisms such as mismatch repair (MMR), base excision repair (BER), nucleotide excision repair (NER) and post replication repair (Nealon, Nicholl et al. 1996; Wood 1996; David, Efrati et al. 1997; Fortini, Pascucci et al. 1998).

The types of DNA modifications caused by spontaneous processes include base loss, base deamination, base alkylation, base oxidation, and single-strand breakage (Kunkel, Schaaper et al. 1983; Storz, Christman et al. 1987; Cooper and Krawczak 1989; Shen, Rideout et al. 1992; Wagner, Hu et al. 1992; Lindahl 1995). Only modifications from base loss, base deamination and oxidative damage will considered in this discussion. Base loss from spontaneous depurination or depyrimidation leading to the

formation of AP (apurinic or apyrimidinic) sites has been observed in mammalian system at physiological temperature and pH (Ehrlich, Norris et al. 1986). AP sites have been shown to block DNA synthesis in vitro, and therefore are lethal to the cell (Boiteux and Laval 1982; Kunkel, Schaaper et al. 1983; Strauss 1985). Occasionally, AP sites are bypassed and adenines are frequently inserted (Schaaper, Kunkel et al. 1983; Kunkel 1984). The insertion of adenine opposite of an AP site has been observed to be dependant on the DNA polymerase used and the sequence context (Cai, Bloom et al. 1993). The insertion of adenine in sites derived from depurination will result in the formation of transversion. The estimated of purine loss is 10 000 per cell generation (Lindahl 1979).

DNA modifications from the deamination of cytosine and methylated cytosine at dinucleotide CpG sites and trinucleotide CpNpG sites are frequently mutated events despite their relative low frequency of sequence occurrence (Cooper and Krawczak 1989). The deamination rate of 5mC was found to be around 9.5×10^{-10} s^{-1} under physiological temperature and pH (Ehrlich, Norris et al. 1986). The deamination of cytosine to yield U and deamination of methylated cytosine to yield T leads to the DNA replication mediated formation of CG to TA (or GC to AT) transition mutations. The 5mC to T transition mutation occurs about 10 times more frequently than other transitions (Holliday and Grigg 1993). In addition, transition mutations at CpG sites have been discovered in large percentages of human germline and somatic diseases caused by mutations; examples are 32% of germline mutations in the low density lipoprotein

receptor gene and 50% of germline mutations in the *p53* gene (Li-Fraumeni syndrome) (Cooper and Krawczak 1990; Rideout, Coetzee et al. 1990).

Damage from oxygen and oxygen radical species are widely believed to a main contributor of spontaneous mutagenesis. It has been estimated that some 10 000 oxidative DNA lesion are produced per human genome per cell per day (Ames 1989). Oxygen derived damage has been shown to be both cytotoxic (Schaaper and Loeb 1981; Schaaper, Kunkel et al. 1983; Moran and Wallace 1985; Laspia and Wallace 1988; Evans 1993) and mutagenic (Halliwell and Aruoma 1991; Ames, Shigenaga et al. 1993). Oxygen derived damage includes the addition of reactive oxygen species to purine and pyrimidine bases, abstraction of hydogens from the deoxyribose sugar and cleavage of the phosphodiester DNA backbone causing single strand breaks (Aruoma, Halliwell et al. 1989; Breen and Murphy 1995). Studies have shown that oxidative derived base damage by the addition of oxygen radicals form stable and unstable products. Stable products that retain the structural features of the purine or pyrimidine are readily bypassed by DNA polymerases in vitro (Ide, Petrullo et al. 1991; Purmal, Kow et al. 1994). The 8-oxo-dG adduct can mispair with any nucleotide and therefore lead to the formation of a variety of mutations (Einolf, Schnetz-Boutaud et al. 1998). Misincorporation of 8-oxo-dG opposite adenine leads to A to C transversions, and misinsertion of dATP opposite template 8-oxo-dG results in G to T transversions (Le Page, Guy et al. 1998). Other common oxygen-related damage base products include thymine glycol, 8-hydroxyguanine and FaPy lesions.

The HPRT gene is widely used in genetic toxicology as a selectable marker in mutation assays (Caskey and Kruh 1979). The enzyme is involved in the reconstitution

of purine nucleotides in the salvage pathways. Cells expressing the HPRT enzyme can utilize the exogenous purine base hypoxanthine or guanine in conjunction with 5'-phosphoribosyl – 1 – pyrophosate (PRPP) leading to the formation of 5' IMP or 5' GMP, respectively (Kornberg, Lieberman et al. 1955). The gene is expressed constitutively at low levels in all tissues (Krenitsky, Papioannou et al. 1969; Kelley and Wyngarrden 1983). The gene is X-linked and is thus present in only one copy per cell in males (Hoefnagel 1965; Rosenbloom, Kelley et al. 1967; Migeon, Der Kaloustian et al. 1968). In addition, it has the characteristic of being a non-essential nuclear gene in vitro, therefore lack of its enzyme activity gives no selective disadvantage (Thilly, DeLuca et al. 1976). Mutant cells lacking an active enzyme can be selected in cell culture with a purine analog, 6-thioguanine (6TG), whose conversion to nucleotide and incorporation into the DNA is lethal to non-mutant cells with functional HPRT enzyme activity (Thilly, Deluca et al. 1978). It appears to be extremely sensitive, mutations giving the 6TG-resistant (6TGR) phenotype have been seen at all 9 of its exons and in more than 300 of the 657 coding region basepairs, indicating that a large fraction of the coding region is critical for enzyme function (Cariello, Douglas et al. 1997). Clinically, total or partial deficiency in HPRT enzyme activity in humans is associated with Lesch-Nyhan syndrome and gouty arthritis. Lesch-Nyhan syndrome patients are characterized by the overproduction of uric acid and nervous system aberrations including mental retardation, spasticity, choreoathetosis, hyperuricemia and compulsive self-mutilation (Seegmiller, Rosenbloom et al. 1967; Stout and Caskey 1985).

A laser-induced fluorescence detection capillary instrument, Constant Denaturant Capillary Electrophoresis, or CDCE (Khrapko, Hanekamp et al. 1994; Khrapko, Coller et

al. 1997) a modification of Denaturant Gradient Gel Electrophoresis (DGGE), uses a constant separation temperature zone to separate sequences based on the slightly different equilibrium constant and secondary structure that results from every sequence change in the low melting region. Faster-moving, unmelted DNA fragments in equilibrium with slower-moving, partially melted forms are separated in the heated portion of the capillary. Within a certain temperature range the position of the melting equilibrium and thus the average electrophoretic mobility of each mutant in different (Khrapko, Hanekamp et al. 1994; Khrapko, Coller et al. 1996). Alterations in the low melting region of the DNA as small as a single basepair substitution can change the melting temperature (T_M) of the partially melted form causing the DNA to characteristically move slower or faster under separation conditions. The basis of this separation allows for the detection of mutant for non-mutant species in a mixed population. CDCE has improved the 5 x 10^{-3} mutation detection limit of DGGE to sensitivity of identifying one mutant sequence in a million wildtype sequence (10^{-6}) and in addition offers better resolution and has significantly reduced the time to visualize mutants (Khrapko, Hanekamp et al. 1994; Khrapko, Coller et al. 1997).

As important as it is to have the ability to detect mutants at a low level ($\leq 10^{-6}$) a particular mutation must be numerically represented with statistical significance in a given sample in order to determine its mutant fraction with acceptable precision. To reduce the 95% confidence interval on the estimate of the mean to within 20%, a particular mutation must be present at least 100 times. Additionally, experimental error is introduced in any physical array and must be considered (Oller, Rastogi et al. 1989).

Therefore, to generate a significant number of mutants required to obtain a "robust" mutational spectrum, cells were grown in large culture (2.4×10^9 cells) for many generations (approx. 60 doublings). These parameters were devised from a mutation assay model (Oller, Rastogi et al. 1989) and should provide the necessary number of independently arising spontaneous mutants to determine reproducible hotspots ($\geq 0.5\%$) in 6TG selected HPRT mutants. Specifically, I hope to determine the spontaneous mutational hotspots that occur at 0.5% or greater in exon 2 and exon 3, high melting region. Since my total HPRT- mutant fraction is $60 \times 2 \times 10^{-7} = 1.2 \times 10^{-5}$, this corresponds to measuring mutant fractions for specific mutants at $5 \times 10^{-3} \times 1.2 \times 10^{-5} \approx 10^{-8}$. HPRT mutants present at a mutant fraction would be expected to have been present in 240 cells prior to 6TG selection.

With this cell culture approach, the improvements in high-fidelity PCR and the low limit of detection of CDCE a clear spontaneous mutational spectrum in the HPRT gene is possible. DNA modifications from endogenous chemicals, DNA polymerase errors or faulty mismatch repair (MMR) systems are frequently hypothesized causes of spontaneous mutations. In order to show the effect that spontaneous pathways are responsible for genetic change one must show that these changes are characteristic in kind and/or frequency. Measuring the spontaneous spectrum in vitro can provide a useful means to compare with spectra measured in vivo and to provide useful contrast with mutagenesis caused by chemical and/or environmental agents, and a better understanding of the predominant mechanisms leading spontaneous mutagenesis.

2. Previous Observations in Human HPRT Gene

2.1. Inherited Mutations

Currently, collections of mutational data from human blood sample and from tumors of various organs are being compiled and human "population" mutational spectra for various genes in several organs is beginning to emerge (Cariello, Douglas et al. 1997; Krawczak and Cooper 1997). It is the hope that the data from these spectra will elicit the primary pathway for mutations seen in people, and therefore provide information regarding the mechanism(s) ultimately leading to the formation of disease. While only a few hundred mutations have been identified and a true "spectra" based on frequency of event cannot be determined it is possible to identify hotspot mutations within the small set of collected mutations. Keeping in mind that these mutants may be biased in that these mutations were selected for in that they cause disease or from loss of function, using Bonferroni inequalities, hotspot mutations can be tentatively identified, by excluding the probability of it being a random event.

One such mutational database focuses on the HPRT gene, where one mutation represents one 6TG selected mutant per person from individuals born with Lesch-Nyhan syndrome or gout, has been developed (Cariello, Douglas et al. 1997). The data from this set will be regarded as the germinal spectrum (see figure 1). To date, 100 mutants have been entered into the germinal database. Of the 100 mutants identified in the germinal database, C to T transition mutations at CpG sites, basepair 151 and 508, appear to be hotspots as derived from Bonferroni inequalities. A second mutation, a C to A transversion at basepair 222 appears also to be hotspot as defined from Bonferroni inequalities.

2.2. Somatic Mutations in T cell Lineage

A separate collection of HPRT mutational data focuses on point mutations that have arisen in T-lymphocytes from healthy individuals (Podlutsky and Lambert 1997). The healthy individuals in this study are non-smoking males that have not been previously diagnosed with a genetic disease (Podlutsky and Lambert 1997). These mutants were combined with the Cariello set include both males and female and smoker and non-smokers. The mutations found in the Cariello set of somatic mutations include normal people, smoker and non-smokers, represent one 6TG selected mutant per person. It is important to note that the somatic mutations may be biased from the 6TG selection process. The data from this combined set will be regarded as the somatic spectrum (see figure 1). To date, 291 mutants have been entered into the somatic database. Of the 291 mutants identified, G to A transitions and G to T transversions mutations at basepair 197 appears to be a hotspot as defined from Bonferroni inequalities. Other hotspots as defined by Bonferroni inequalities include G to A transition mutation at basepair 3 and 617, and a C to T transition mutation at basepair 508, a CpG site. A C to T transition mutation at basepair 551 appears to be a hotspot was not a CpG site.

There are some similarities between the two databases as some of the mutants come from the same papers (Burkhart-Schultz, Thompson et al. 1996). Careful examination of the databases allowed only one mutation per person to be added. As more mutations are added more hotspot areas will be discovered. As it stands, about 25-30% of the mutations that arose germinally and somatically is found in point mutation hotspot

areas. Of the hotspots found in both databases, only the C to T transition at basepair 508

was found to be a hotspot in both.

Figure 1: Comparison between germinal and somatic in vivo mutational databases.
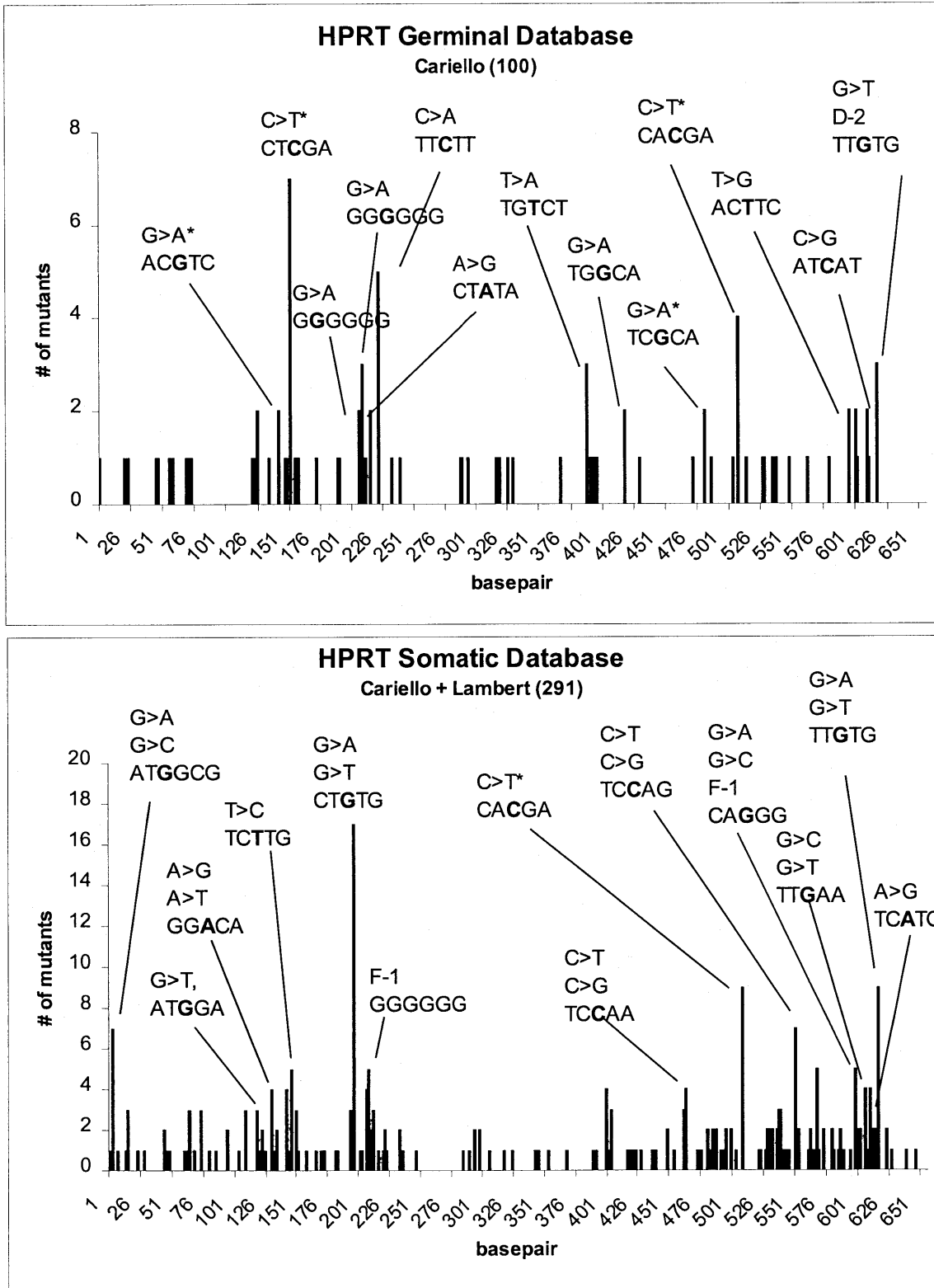
2.3. Mutations in Mammalian Cells in Culture, Spontaneous and Induced

2.3.1 Human Cells

In human B-lymphoblastoid cells, a point mutational spectrum was determined to discover if reactive oxygen species were responsible for spontaneous mutagenesis seen in exon 3 of the human HPRT gene (Oller and Thilly 1992). The region examined discovered two small hotspots (~1%) in the low melting region of exon 3. The two hotspots identified in all three cultures were both deletions, a deletion of A at basepair position 256 or 257 (0.9% of the 6TGR point mutations) and a deletion of two consecutive Gs at basepair position 237-238 (0.7% of the 6TGR point mutations). Despite finding several other point mutations present in only one or two of the cultures, no common mutations were found after the examination of the high melting region of exon 3 (Oller and Thilly 1992). Additionally, it was determined from this report that spontaneous point mutations found in exon 3 were not derived from oxygen-related pathways (Oller and Thilly 1992).

In a separate experiment measuring point mutations in exon 3 of the HPRT gene after exposure to 254-nm UV-light, three 6TGR mutants were identified in the untreated cultures. Of the three mutants identified, two were found in the high melting region of exon 3, a 12-bp deletion at position 192/193-288/289 (2.5% of the 6TGR point mutations) and a AT to TA transversion at position 205 (~2% of the 6TGR point mutations). The third mutant, a 12-bp deletion was found in the low melting region of exon 3 at position 280-291 (0.5% of the 6TGR point mutations) (Keohavong, Liu et al. 1991). None of these three mutants found were seen in a previous experiment (Oller and

Thilly 1992). Isolates of individual mutants clones were analyzed and sequenced for the spontaneous mutational spectrum of TK-6 cells and on CHO cells (Zhang, Vrieling et al. 1992; Lichtenauer-Kaligis, Thijssen et al. 1993; Lichtenauer-Kaligis, van der Velde-van Dijke et al. 1993; Lichtenauer-Kaligis, Thijssen et al. 1995; Lichtenauer-Kaligis, Thijssen et al. 1996). However, in both cases, due to the small numbers of mutants sequenced, a point mutational spectrum with statistical significance cannot be determined.

The spontaneous spectra in MT-1 cell line, a mismatch-repair deficient cell line with a high mutation rate (~30X higher than TK-6) was determined (Goldmacher, Cuzick et al. 1986). MT-1 is a mutant derivative of the TK-6 cell line, was established after ICR-191 treatment, and it resistant to killing by the alkylating agent MNNG (Goldmacher, Cuzick et al. 1986). Two mutations were found in the untreated culture for exon 2. A G to T transversion mutation at basepair 40 (4% of the 6TGR point mutations) resulting in a Glu to STOP and deletion of a G at basepair position 46 (1% of 6TGR point mutations) resulting in a frameshift. Of the 5 spontaneous mutation found in exon 3, only one mutation, a +G frameshift mutation (13% of the 6TGR point mutations) was located in the high melting region (Kat 1992). The +G frameshift mutation found in a run of six guanine residues is consistent to what is seen in microsatellite repeat areas in mutator cell lines (Bhattacharyya, Ganesh et al. 1995; Malkhosyan, McCarty et al. 1996; Ohzeki, Tachibana et al. 1997).

Figure 2: Comparison of mutational spectra for HPRT exon 3 for human TK-6 cells.

The mutational spectra for HPRT exon 3 for human TK-6 cells has been determined for ICR-191 (Cariello, Keohavong et al. 1990); MNNG Ling, L, unpublished; (Keohavong, Liu et al. 1991); BPDE (Keohavong and Thilly 1992) and Spontaneous (Oller 1989). The spontaneous mutational spectrum also includes found in this assay and from A. Tomita-Mitchell, unpublished. The kind, position and frequency of the induced mutational spectrum are different from one another and different from the TK-6 spontaneous mutational spectrum. Indicating that the primary for mutations in the induced sets did not derive from spontaneous pathways.

Mutational Spectra For HPRT Exon 3 For Human TK-6 Cells

ICR-191

+G

30
20
10
0

MNNG

G->A

15
10
5
0

PERCENTATAGE OF 6TGR MUTANTS

UV

G->A   T>G   T>C

T>G

3
2
1
0

BPDE

G->T   G>C -1G

G>A

A>T -A

4
2
0

SPONTANEOUS

?   -GG   -A/-AT   -A

2
1
0

135   155   175   195   215   235   255   275   295   315

BASEPAIR POSITION
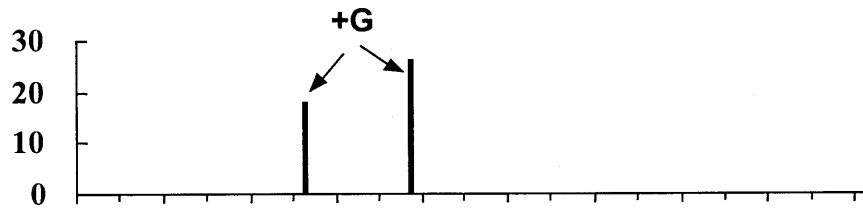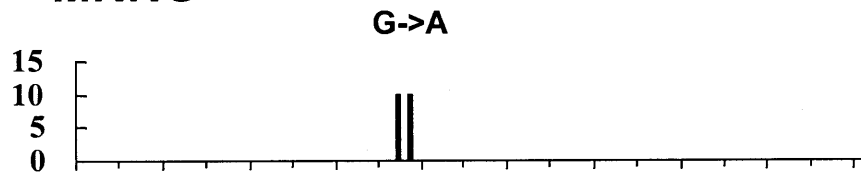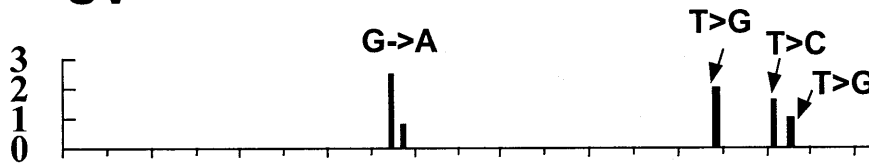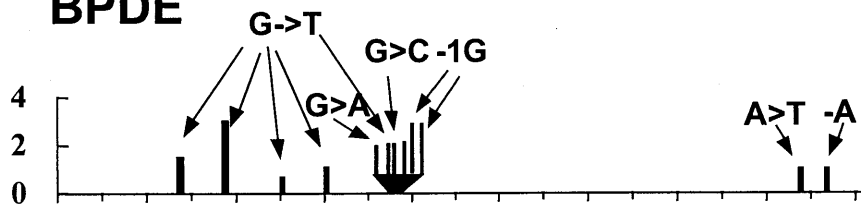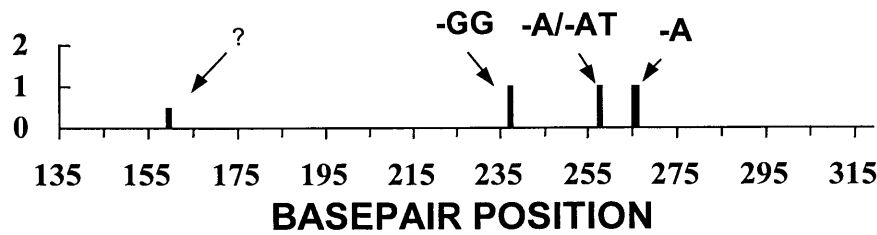
2.4. Mutations induced by DNA polymerases

DNA polymerases have been studied and identified as a source of spontaneous mutagenesis in vivo and in vitro (Loeb, Liu et al. 1983; Kunkel 1985; Kunkel, Alexander et al. 1986; Roberts and Kunkel 1988; Thomas, Roberts et al. 1990). DNA polymerases induce mutation either by direct misincorporation of a non-complementary nucleotide into a template strand, misalignment or strand-slippage, or misrepair/misreplication of an erroneous nucleotide (Kunkel 1986). Despite the various ways in which DNA polymerases can induce mutations, DNA polymerases replicate the 6×10^9-nucleotide human genome with relatively high precision incorporating on average only one incorrect nucleotide to 10^5 to 10^6 correct nucleotides (Kunkel and Loeb 1981; Roberts and Kunkel 1988; Thomas, Roberts et al. 1990; Thomas, Roberts et al. 1991). Since the average spontaneous mutation frequency per basepair is on the order of 10^9 to 10^{11} mutations per bases replicated per generation (Drake 1970), DNA polymerases are only partly responsible for maintaining the integrity of the genome. The extremely high fidelity of replicating the human genome is maintained by, first, selectively incorporating the correct nucleotide into a growing replication fork. The process of incorporating correct nucleotides is governed by free energy, where the difference between correct and incorrect nucleotides result in approximately on error in 100 nucleotides (Loeb and Cheng 1990). If an incorrect base is misincorporated into the template strand, a $3' \rightarrow 5'$ exonuclease can excise the incorrect base before the polymerase can extend pass the mistake. Errors that are not corrected by this proofreading mechanism may be corrected

by post-replication repair, which selectively removes errors from the newly synthesized strand (David, Efrati et al. 1997).

The types of errors seen as the result of DNA polymerase are most frequently frameshift mutations by the addition and deletions of a small number of nucleotides by the slippage of the polymerase during replication. As hypothesized by (Streisinger, et al., 1966) polymerase slippage can occur on either strand, and misaligned intermediate generated unpaired nucleotides in either the template or the primer strand. DNA polymerases may also induce single basepair substitutions and large deletions.

3.   Experimental Plan and Procedures

3.1.   Obtaining an Unbiased Sample of Spontaneous Mutants

In order to obtain an unbiased sample for measuring spontaneous mutations in the human nuclear HPRT gene, the following experimental protocol was followed. Cells were grown for many generations (approximately 60 doublings) in large bulk culture (6 liters) to generate a significant number of spontaneous mutants under pristine laboratory conditions. Mutants cells resistant to 6-thioguanine (6TG) were selected. DNA was isolated from 6TG-selected and unselected cells. The unselected DNA samples will serve as the 'background' mutants due to polymerase errors or artifacts from the experimental procedure. Desired sequences on interest were directly amplified from 6TGR mutant cells using high-fidelity PCR with native *Pfu*™ DNA polymerase. Native *Pfu*™ DNA polymerase is a thermostable DNA polymerase enzyme that has an average error rate of 6.5×10^{-7} mutations per basepair duplication (Andre, Kim et al. 1997). Mutant sequences were separated and enriched from non-mutant sequences in heteroduplex fractions using

CDCE. The heteroduplex fraction was amplified and mutants were displayed as homoduplexes. Spontaneous 6TGR mutants with a mutant fraction of 5×10^{-3} or higher were examined.

CDCE enrichment for mutants in a target sequence can be performed as either homoduplexes or heteroduplexes. Mutants are first transformed into heteroduplexes with wild-type DNA by simply boiling and reannealing the DNA sample. All heteroduplexes have a lower melting temperature since the mismatch of the mutant with the wildtype creates a less thermodynamically stable molecule with lower electrophoretic mobility. Thus, all mutants can be enriched for at once because they will all move slower than the wild-type and by allowing the faster moving wild-type molecule elute off the capillary column, the heteroduplex fraction can be collected into an Eppendorf tube with 4 µl of 0.1xTBE$^{1/2}$ with 0.1mg/ml bovine serum albumin. Heteroduplex enrichment simplifies the mutant enrichment procedure because the sample is a complex mixture of mutants and a temperature can be determined where all heteroduplexes will run closely together and can thus be collected in one fraction. After heteroduplex separation, homoduplex separation then can be used to isolate individual mutants for the purpose of sequencing and identification.

Figure 3: Protocol for experimental design.

```
┌──────────────────────────────┐
│   GROW CELLS IN LARGE        │
│   CULTURE (6 LITERS) FOR     │
│   MANY GENERATIONS           │
│   (APPROX. 60 DOUBLINGS)     │
└──────────────┬───────────────┘
               │
               ▼
┌──────────────────────────────┐
│   SELECT FOR MUTANTS         │
│   USING 6-THIOGUANINE        │
└──────────────┬───────────────┘
               │
               ▼
┌──────────────────────────────┐
│   ISOLATE DNA FROM           │
│   SELECTED AND UNSELECTED    │
│   CELL                       │
└──────────────┬───────────────┘
               │
               ▼
┌──────────────────────────────┐
│   DIRECTLY AMPLIFY 6TGR      │
│   MUTANTS USING HIFI-PCR     │
└──────────────────────────────┘
```

SEPARATION OF MUTANTS FROM NON-MUTANTS USING CDCE

CDCE/PCR ENRICHMENT OF MUTANT SEQUENCES

IDENTIFY SPONTANEOUS MUTANTS OCCURING AT A MF = 10^{-3} OR HIGHER

ISOLATE/SEQUENCE SPONTANEOUS 6TGR MUTANTS
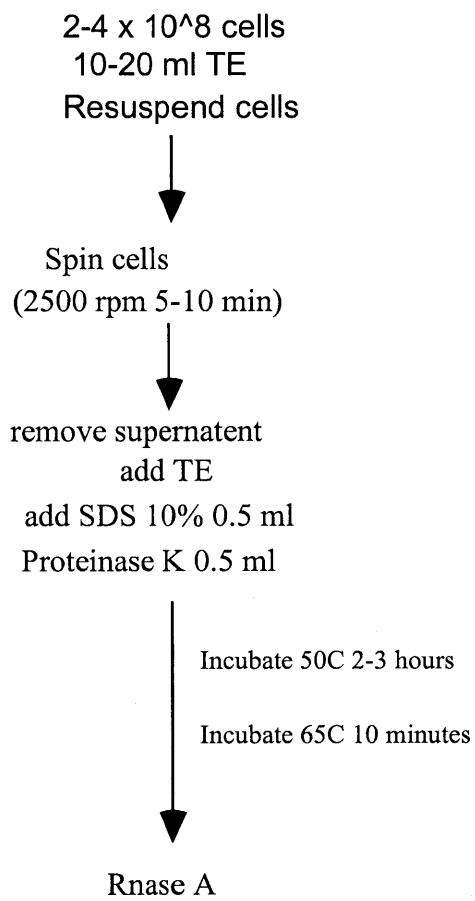
Cells were grown in RPMI 1640 medium (Gibco Labs, Grand Island, NY), supplemented with 5% horse serum (Gibco Labs, Grand Island, NY). Suspension cultures were maintained in 6 liters spinner flasks (tanks) in a 37°C incubator with 5% CO_2. Air was sparged in at 500 ml/min provided oxygen. Cultures were diluted daily to 4×10^5 cells/ml. Three cultures were started with 10^3 cells from a stock culture that had a mutant fraction of 2×10^{-6}. This way, the probability of having a pre-existing mutant was 0.2% ($10^3 \times 2 \times 10^{-6}$). Furthermore, a mutant present at that point would be detected at a MF of 10^{-3}. Mutant fractions were measured several times during expansion of the cultures. When the culture reached 2.4×10^9, the average MF was 2×10^{-6}. There were on average 48000 spontaneous 6TGR mutants in each culture at the time of selection with 6TG. After cultures reached 2.4×10^9 cells (4×10^5 cells/ml in six liters) exponential growth was maintained for 48 days before selection. Mutant fractions and plating efficiency was determined by plating with and without 6TG (Sigma Chemical Co., St. Louis, MO.) every 6 days. Due to the phenotypic lag associated with HPRT resistance to 6TG selection, it is expected that only those mutants present by day 42-44 would be represented among the HPRT- mutants (Thilly, Deluca et al. 1978). Each culture had a linear increase in the HPRT mutant fraction as a function of generations we can estimate a mutation rate by least squares linear (Furth, Thilly et al. 1981). The doubling time for tanks 1 and 3 were estimated to be around 17-18 hours. Tank 2 slowed down and was not be used for spectra analysis. On day 48, 6TG was added to a final concentration of 1μg/ml to select for HPRT mutants. After cells had resumed exponential growth they were harvested for DNA analysis.

After cells had resumed exponential growth they were harvested for DNA analysis. DNA was isolated from Day 0 Tanks 1, 2, and 3, from both 6TG selected and unselected cells, and from Day 48 Tanks 1, 2, and 3, again from both 6TG selected and unselected cells. DNA was extracted by the following method chosen as a safe way to isolate DNA without creating chemical artifacts on the DNA in the process (Khrapko, Coller et al. 1997):

Figure 4: DNA isolation procedure.

2-4 x 10^8 cells
10-20 ml TE
Resuspend cells

Spin cells
(2500 rpm 5-10 min)

remove supernatent
add TE
add SDS 10% 0.5 ml
Proteinase K 0.5 ml

Incubate 50C 2-3 hours

Incubate 65C 10 minutes

Rnase A

Primers previously determined by (Kat 1992) for examination of the HPRT gene using DGGE were synthesized by Synthetic Genetics (San Diego, CA). Genomic DNA was quantified by UV spectrometry and with an internal standard. Each 50 or 10 µl PCR reaction will be performed inside closed glass capillaries using an Air Thermo Cycler™ (Idaho Technologies). Native *Pfu*™ DNA polymerase was obtained from Stragene (La Jolla, CA). The 2'-deoxynucleoside-5'-triphosphates (dNTPs) were purchased from Pharmacia (Picataway, NJ). Each PCR reaction mixture contained 20 mM Tris; 10 mM KCL; 6 mM (NH4)2SO4; 0.1% Triton X-100; 2 mM MgCl2; 150 µM each dNTP; 100 mg/ml nuclease free bosine serum albumin; 0.2 µM each primer; and 1 unit of *Pfu*™ DNA polymerase. The reaction mixture pH was 8.5 at 25°C but 95°C the pH should be around 6. PCR was carried out in two rounds. Each cycle in the first round of the PCR reaction consisted of 15 seconds at 95°C for DNA template denaturation, 30 seconds at 45°C for template-primer hybridization and 30 seconds at 72°C for DNA polymerization. After cycling, the PCR mixture was incubated at 72°C for 2 minutes and at 45°C for 30 minutes to allow for extension of unhybridized fragments. Similar conditions were used for the second round of PCR. Each cycle in the second round of the PCR reactions consisted of 10 seconds at 95°C for DNA template denaturation, 30 seconds at 45°C for template-primer hybridization and 30 seconds at 72°C for DNA polymerization. Again, the PCR mixture was incubated at 72°C for 2 minutes and at 45°C for 15 minutes to allow for extension of unhybridized fragments.

The exon 2 sequence was amplified from genomic DNA for 30 cycles in 50-µl reaction mixture using unlabeled primers TW1 and TW2 which generated a 131-bp

fragment (figure 5). The 131-bp fragment contains the 107-bp coding region of exon 2 (bp 28-134) along with 14 bp of 5' and 10 bp of 3' flanking intron (Kat 1992). Then a second round of PCR was performed for 30 cycles in 10-μl reaction mixture with the 131-bp fragment as a template using 5'-FITC end labeled clamp, GC TW3 and primer TW1 (figure 5). The amplified sequence using primers, GC TW3 and TW1, isolated 71-bp of the 107-bp coding region of exon 2 (Kat 1992). The resultant 151-bp fragment was analyzed by CDCE to detect spontaneous mutations occurring within 71-bp of exon 2. The total expected PCR noise is to be 2.3×10^{-3} mutations per basepair per doubling (Andre, Kim et al. 1997). The PCR efficiency for primers GC TW3 and TW1 was approximately 50-55%.

Figure 5: Primer sequences used for the analysis of 6TGR mutation in exon 2.

Primers, TW1 and TW2, served to amplify exon 2 from genomic DNA. The TW1 and TW2 fragment was reamplified with the 60-bp primer, GC TW3, adding a 40-bp clamp sequence 3' to the coding region of exon 2. The TW1/GC TW3 fragment was suitable for analysis for CDCE. The sizes of the TW1/TW2 and GC TW3 fragments are given below the layout diagram. TW3 corresponds to the exon 2-specific section of GC TW3 and was used for sequence analysis of exon 2 alterations.

EXON 2 PRIMERS USED FOR 6TGR ANALYSIS

PRIMER SEQUENCES

TW 1   5' ATA TTT CTT TTC AGA TTA GT 3'

TW2   5' TCT TAC TTA CCT GTC CAT AA 3'

TW 3   5' TTA GTC CAT GAG GAA TAA AC 3'

GC TW 3   5' CGC CCG CCG CGC CCC GCG CCC
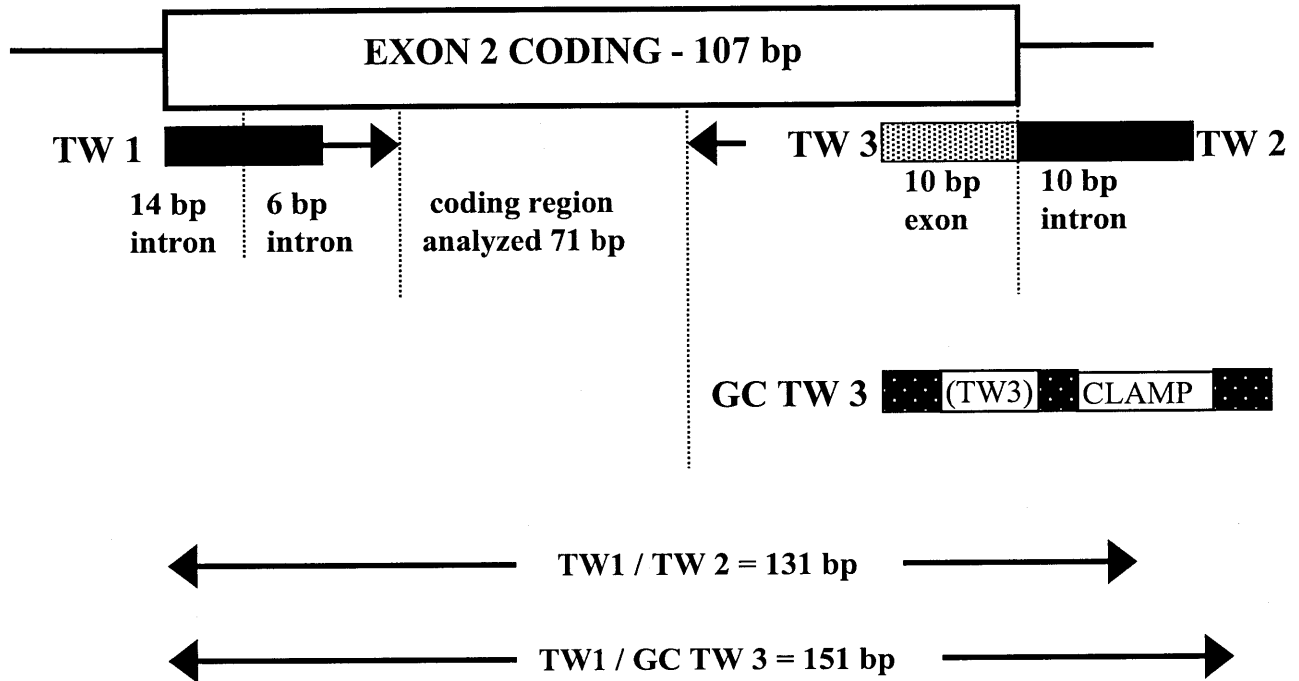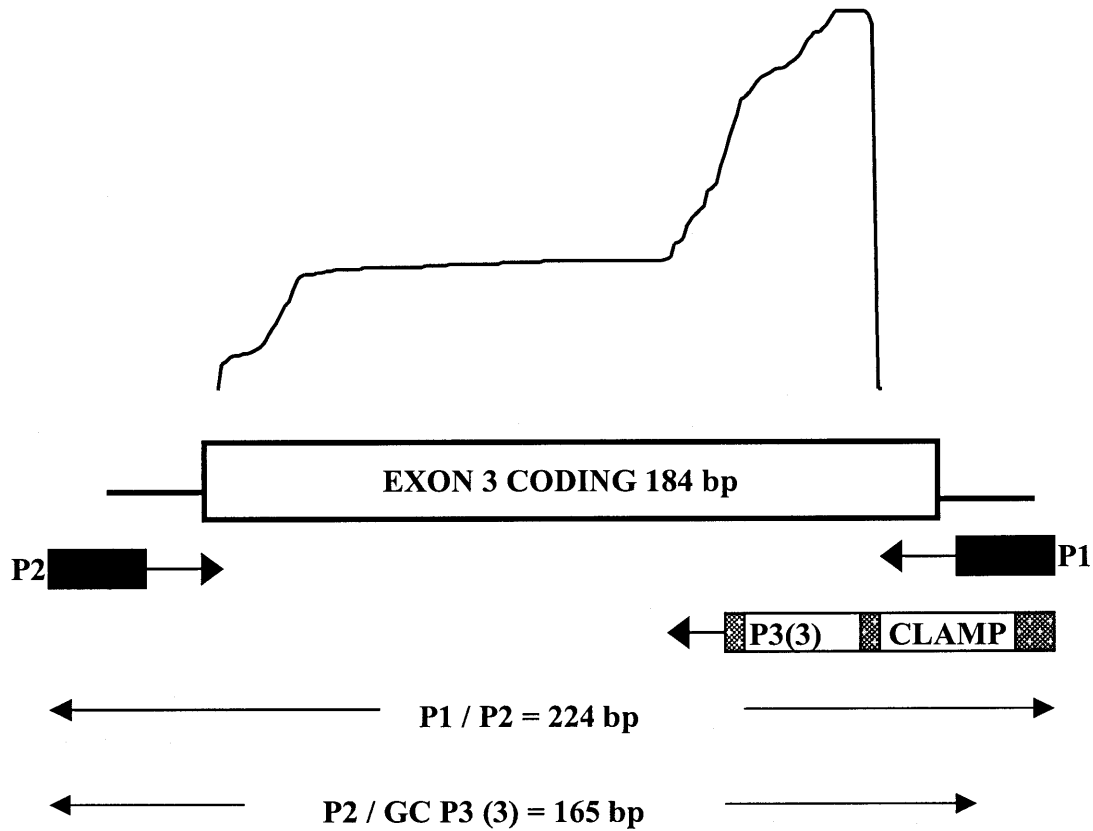GTC CCG CCG CCC CCG CCC GTT AGT
CCA TGA GGA ATA AAC 3'

The exon 3 sequence was first amplified from genomic DNA for 30 cycles in 50-μl reaction mixture using unlabeled primers P1 and P2 which generated a 224-bp fragment (figure 6). Then a second round of PCR was carried out for 30 cycles in 10-μl reaction mixture with the 224-bp fragment as a template using 5'-FITC end labeled clamp P3 (3) and an internal primer, P2 (figure 6). Amplification with the primers, GC P3 (3) and P2, served to isolate the high melting region [Kat, 1992 #66; Oller, 1992 #28; (Cariello, Keohavong et al. 1990). The primer sequences previous described in (Cariello, Keohavong et al. 1990; Keohavong, Liu et al. 1991; Kat 1992; Oller and Thilly 1992; Okinaka, Anzick et al. 1993) produced a preferential amplified product during this assay and prevented the analysis of exon 3, high melting region. To circumvent this problem, the clamp sequence was placed on the P3 primer, while still maintaining the original sequences context for the P3 and P2 primers. The resultant 165-bp fragment, which now contained a new high-temperature melting region, was analyzed by CDCE to detect spontaneous mutations occurring within the 80-bp formerly high melting region. The expected total PCR noise is to be 2.3×10^{-3} mutations per basepair per doubling (Andre, Kim et al. 1997). The estimated PCR efficiency for primers, GC P3 (3) and P2 was 45-50%.

Figure 6: Primer sequences used for the analysis of 6TGR mutation in exon 3, high melting region.

Primers, P1 and P2, served to amplify exon 3 from genomic DNA. The P1 and P2 fragment was reamplified with the 60-bp primer, GC P3 (3), adding a 40-bp clamp sequence to isolate the high melting region of exon 3. The P2/GC P3 (3) fragment was suitable for analysis for CDCE. The sizes of the P1/P2 and GC P3 (3) fragments are given below the layout diagram. P3 (3) corresponds to the exon 3-specific section of GC P3 (3) and was used for sequence analysis of exon 3, high melting region alterations.

EXON 3 PRIMERS USED FOR 6TGR ANALYSIS
WITH CLAMPED FRAGMENT

PRIMER SEQUENCES

P1   5' CAT ATA TTA AAT ATA CTC AC 3'

P2   5' TCC TGA TTT TAT TTC TGT AG 3'

P3   5' TCC AGC AGG TCA GCA AA 3'

GC P3 (3)   5' CGC CCG CCG CGC CCC GCG CCC
           GTC CCG CCG CCC CCG CCC GTA
           ATC CAG CAG GTC AGC AAA3'

3.2. Mutational Spectrometry

3.2.1. Exon 2

Exon 2, the second largest exon of the HPRT gene is 107 bp, comprising 16% of the coding region. Analysis of the predicted melting profile for exon 2 shows that the exon does not posses a naturally occurring high and low melting region suitable for analysis of CDCE (MacMelt™; BIORAD, Hercules, CA). However, it is possible to alter the melting characteristics of a DNA fragment by addition of a GC-rich sequence, termed a clamp (Myers, Fischer et al. 1985; Myers, Fischer et al. 1985; Sheffield, Cox et al. 1989; Cariello, Keohavong et al. 1990). The clamp alters the melting properties of the fragment; this change greatly increases the fraction of possible mutations that is detectable (Abrams, Murdaugh et al. 1990). Although many of the sequences desired for CDCE analysis do not have a naturally occurring high melting domain, a G+C rich sequence, or a clamp (Myers, Fischer et al. 1985; Myers, Fischer et al. 1985; Sheffield, Cox et al. 1989) can be attached to a target sequence during PCR for CDCE analysis. The clamp vastly increases the number of sequences that can be used for mutational spectral analysis on CDCE. The resulting 151-bp clamp fragment includes 71-bp (66%) of the coding region of exon 2 and 6-bp of the 5' flanking intron was analyzed for spontaneous 6TGR mutants.

The calculated melting map for the clamped wildtype sequence of exon 2 is given in figure (7) (MacMelt™; BIORAD, Hercules, CA). The internal standard constructed for quantitative analysis of exon 2 is TW4 (mutant TW1 primer). TW4 is a single basepair substitution of A to G at basepair position 39 (cDNA position) (see figure 7).

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The predicted melting temperature of TW4 is more stable than the wildtype sequence and therefore, migrates at a faster rate on a CDCE column under separation conditions (MacMelt™; BIORAD, Hercules, CA). Amplifying the TW1 and TW2 fragment with GC TW3 primer and internal standard TW4 primer produced the internal standard. The internal standard will be used to quantify the percentage of hotspot found in exon 2.

The internal standard TW4 and wildtype homoduplexes were mixed together in a ratio approximately 1:1 (WT: IS). The internal standard/wildtype homoduplex mixture was boiled and reannealed to create four peaks: wt: wt homoduplex, is: is homoduplex, and two wt: is heteroduplexes. The boiled and reannealed mixture was analyzed on CDCE with increasing temperature to find the optimal separation temperature. The optimal separation temperature was found to be 68.8°C. All samples were analyzed at the temperature for the presence of spontaneous 6TGR mutants in exon 2.

In the electropherograms of CDCE analysis shown in figure (8), demonstrates the separation and detection at 68.8°C of a mutant fraction of 10^{-2} and 10^{-3}, respectively. A mutant fraction of 10^{-2} appears as a clear sharp peak well above the background. A mutant fraction of 10^{-3} appears as a small peak visible above the background and a mutant fraction of 10^{-4} is obscured by DNA polymerase noise.

Figure 7: Melting profile including internal standard primers for HPRT exon 2 with a 40-bp clamp.

A to G

WT

TW1

TW4(mut)   G

GC TW3*
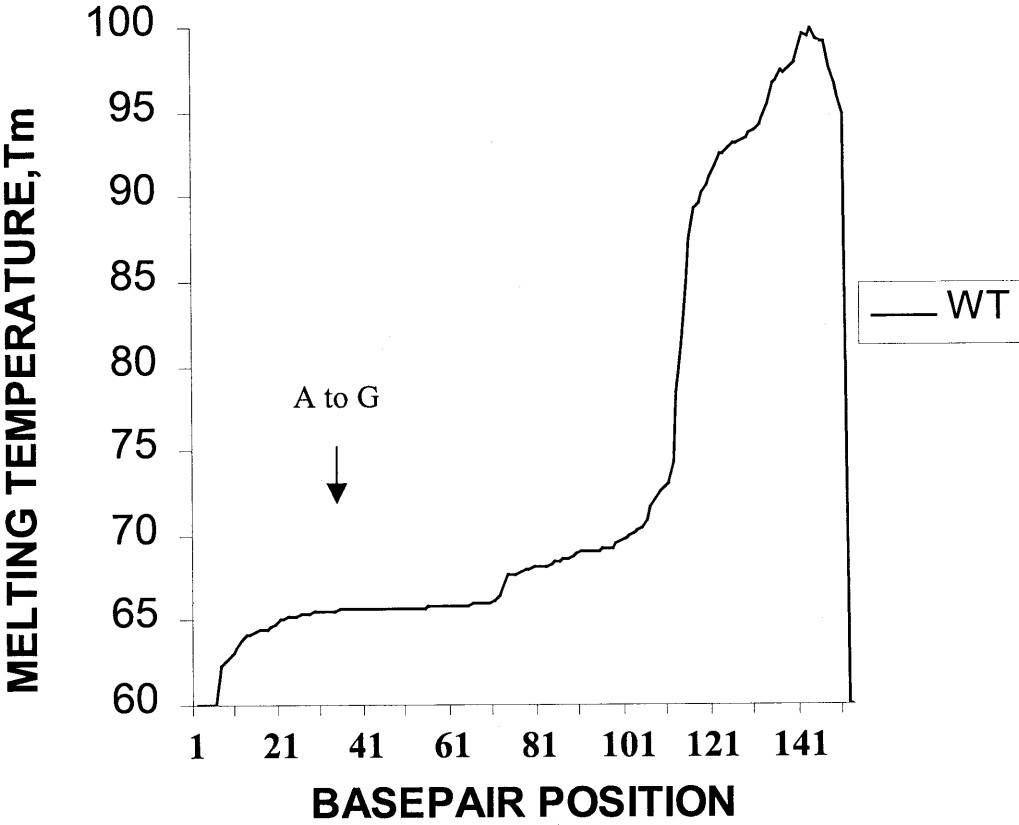
* 5' – flourescein label

Figure 8: Demonstration of sensitivity and detection of 10^{-2} versus 10^{-3} for exon 2.
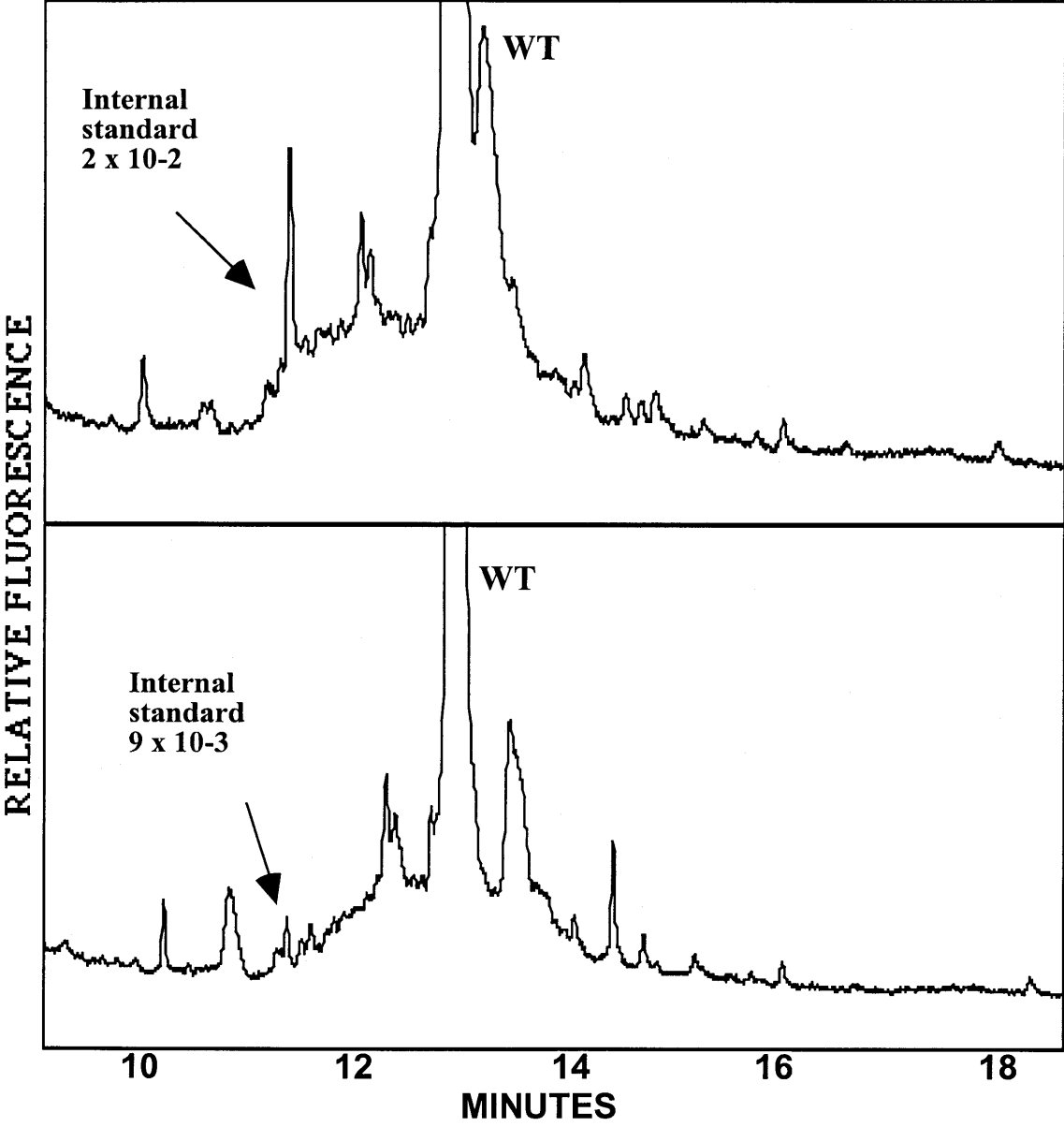
3.2.2. Exon 3, High Melting Region

Exon 3 has a naturally occurring, low-temperature melting region of 104 basepair (bp 215-318) and a high-temperature melting region of 80 basepairs (bp 135-214). Just as in DGGE, CDCE can only detect alterations in the low-temperature melting region of a molecule. The addition of a clamp to the high melting region of exon 3 creates new higher temperature melting region. Mutations present in basepairs 135-214 can now be detected by CDCE, where previous studies have used DGGE (Cariello, Keohavong et al. 1990; Keohavong, Liu et al. 1991; Kat 1992; Keohavong and Thilly 1992; Oller and Thilly 1992; Okinaka, Anzick et al. 1993; Cariello, Cui et al. 1994).

The calculated melting map for the wildtype sequence of exon 3, high melting domain is given in figure (9) (MacMelt™; BIORAD, Hercules, CA). The internal standard constructed for quantitative analysis of exon 3, high melting domain is P3M3. P3M3 is a single basepair substitution of C to T at basepair 222 (cDNA position) (see figure 9). The predicted melting temperature of P3M3 is less stable than the wildtype sequence and therefore, migrates at a faster rate on a CDCE column under separation conditions (MacMelt™; BIORAD, Hercules, CA). Amplifying the P1 and P2 fragment with P2 primer and internal standard P3M3 primer, followed by attachment of the clamp with GC P3 (3) primer, produced the internal standard. The internal standard will be used to quantify the percentage of hotspot found in exon 3, high melting domain.

The internal standard, P3M3, and wildtype homoduplexes were mixed together in a ratio of approximately 1:1 (WT: IS). The internal standard/wildtype mixture was boiled and reannealed to create four peaks: wt: wt homoduplex, is: is homoduplex, and two wt: is heteroduplexes. The boiled and reannealed mixture was analyzed on CDCE with

increasing temperature to find the optimal separation temperature. The optimal separation temperature was discovered to be 74.4°C. All samples will be analyzed at this temperature for the presence of spontaneous 6TGR mutants in exon 3, high melting domain.

In the electropherograms of CDCE analysis shown in figure (10), demonstrates the separation and detection at 74.4°C of a mutant fraction of 10^{-2} and 10^{-3}, respectively. A mutant fraction of 10^{-2} is clearly detected as a sharp peak well above the background, a mutant fraction of 10^{-3} appears as a small peak just above the background and a mutant fraction of 10^{-4} is obscured by DNA polymerase noise.

Figure 9: Melting profile including internal standard primers for exon 3, high melting region with a 40-bp clamp.
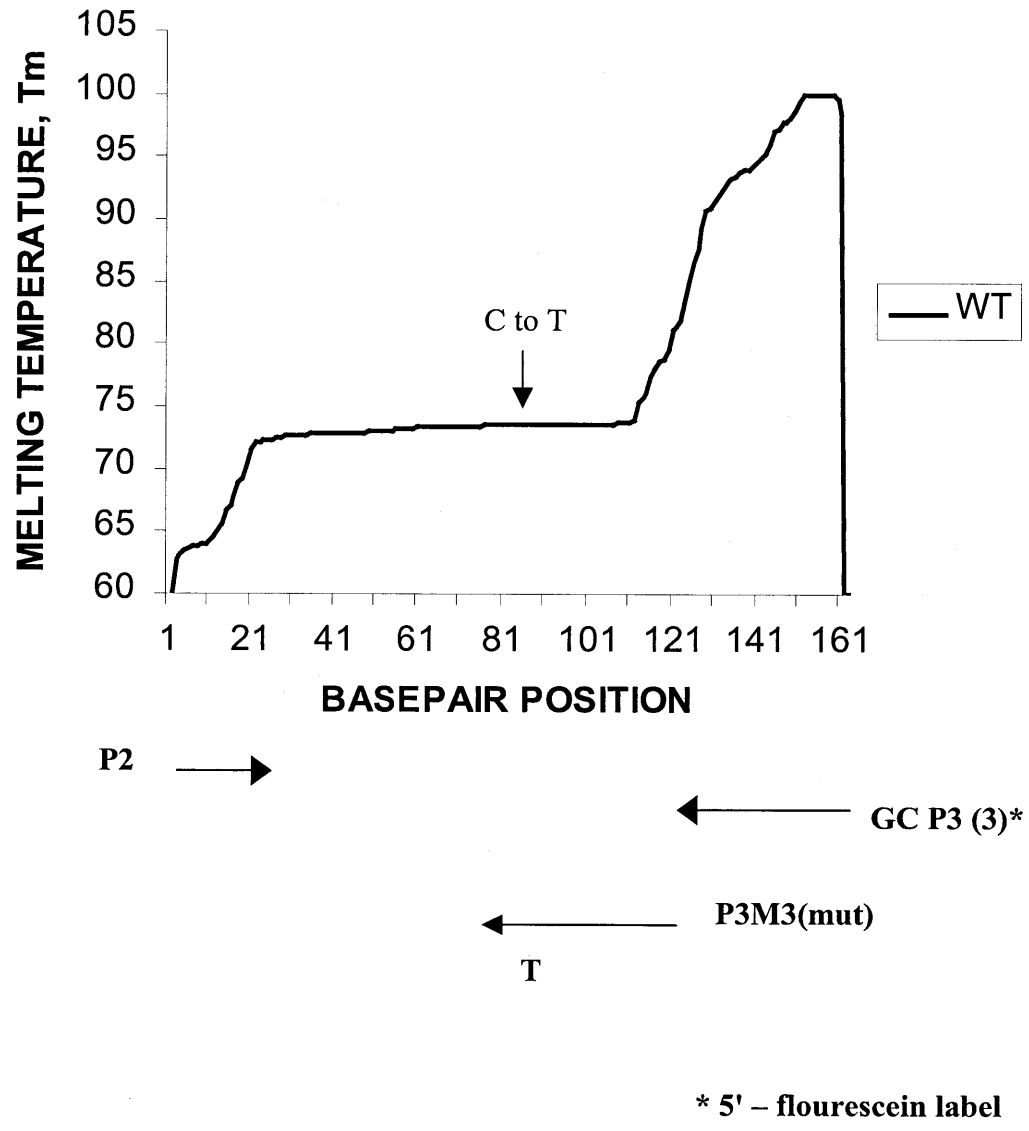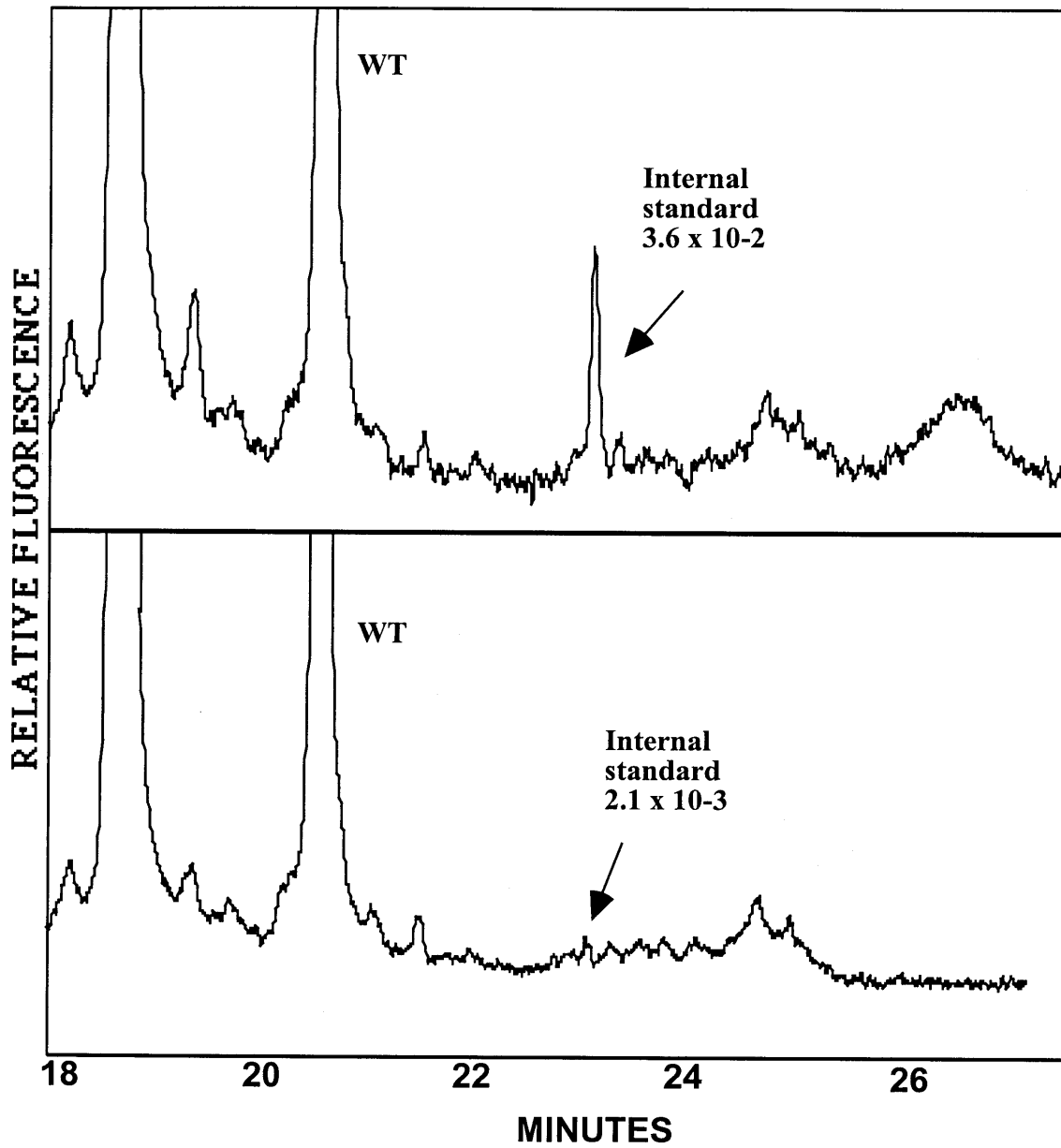
Figure 10: Demonstration of sensitivity and detection of 10^{-2} versus 10^{-3} for exon 3, high melting region.

3.3. CDCE

The CDCE instrumentation used was similar to that described previously (Khrapko, Coller et al. 1997) with a few modifications. Electrophoresis was performed in 75 μm I.D., 350 μm O.D. pre-coated capillaries filled with 5% linear polyacrylamide in TBE$^{1/2}$ (89 mM Tris, 89 mM boric acid and 0.5 mM EDTA, pH 8.3). The polyacrylamide gel in the capillary was replaced prior to each run. PCR products were diluted ten times and loaded with approximately 10^8 copies of amplified DNA fragments. A portion of the capillary was heated by a water jacket connected to a constant temperature circulating water bath. The length of the jacket was 10 cm. To detect DNA, a 488-nm argon laser illuminated the capillary and the emitted light was collected at a right angle by a microscopic objective. This light was directed through two filters, 520-nm bandpass and 530-nm long pass, into a photomultiplier. A current preamplifier as recorded by the Workbench^{TM} data acquisition system (Strawberry Tree, Inc. Sunnyvale, CA), amplified the signal from the photomultiplier.01t3b0s12v1P

4.   Results

4.1.   Analysis of Exon 2

The electropherograms of CDCE analysis in figure (11) demonstrates the heteroduplex separation from wildtype. The electropherogram includes a BPDE treated sample, which serves a positive control for detection of mutant in the exon 2. The electropherograms of CDCE analysis compares the presence of wildtype/mutant heteroduplexes under separation conditions. There does not appear to be any

heteroduplex peaks present in the day 48 samples from Tank 1 and 3 that are different from the day 0 background samples at mutant fraction of 5×10^{-3} prior to enrichment.

The electropherograms of CDCE analysis in figure (12) illustrates the heteroduplex fraction with an internal standard at a mutant fraction of 10^{-2} that will be collected and enriched. The internal standard will allow for the measurement of enrichment. The collected fraction will be amplified and displayed as homoduplexes for identification and quantification of spontaneous 6TGR mutants present in exon 2. Figure (13) illustrates the position of the internal standard heteroduplex in the collected fraction.

The electropherograms of CDCE analysis in figure (14) demonstrates the CDCE separation of wildtype and mutant homoduplex peaks at 68.8°C for exon 2 after CDCE/PCR enrichment. After 60 doublings, tank 3 demonstrated a mutant peak "a" at a fraction of 2.2×10^{-2}. This mutant was not present in tank 1. Tank 1 demonstrated a mutant "b" at a mutant fraction of 1×10^{-2}. However, this mutant also was not reproduce in tank 3.

Figure (15) includes the day 0 selected electropherograms for both tank 1 and tank 3. Tank 3 day 0 appear to have an small mutant present at a mutant fraction of 0.45%.

Figure 11: Demonstration of CDCE separation of heteroduplex region for exon 2 without internal standard.

Figure 12: Collection for heteroduplex fraction for exon 2 with internal standard at mutant fraction of 10^{-2} (Full Scale).
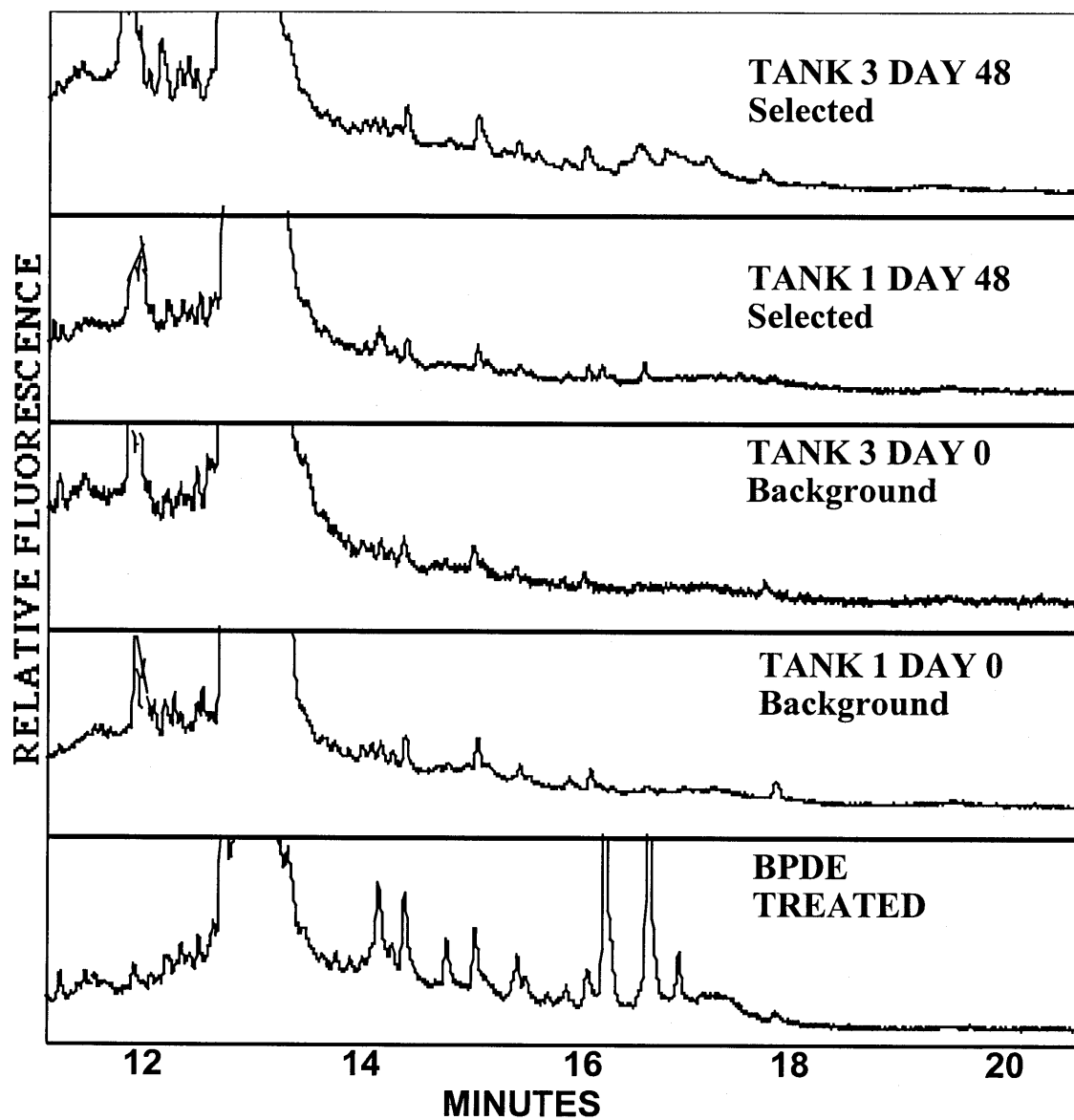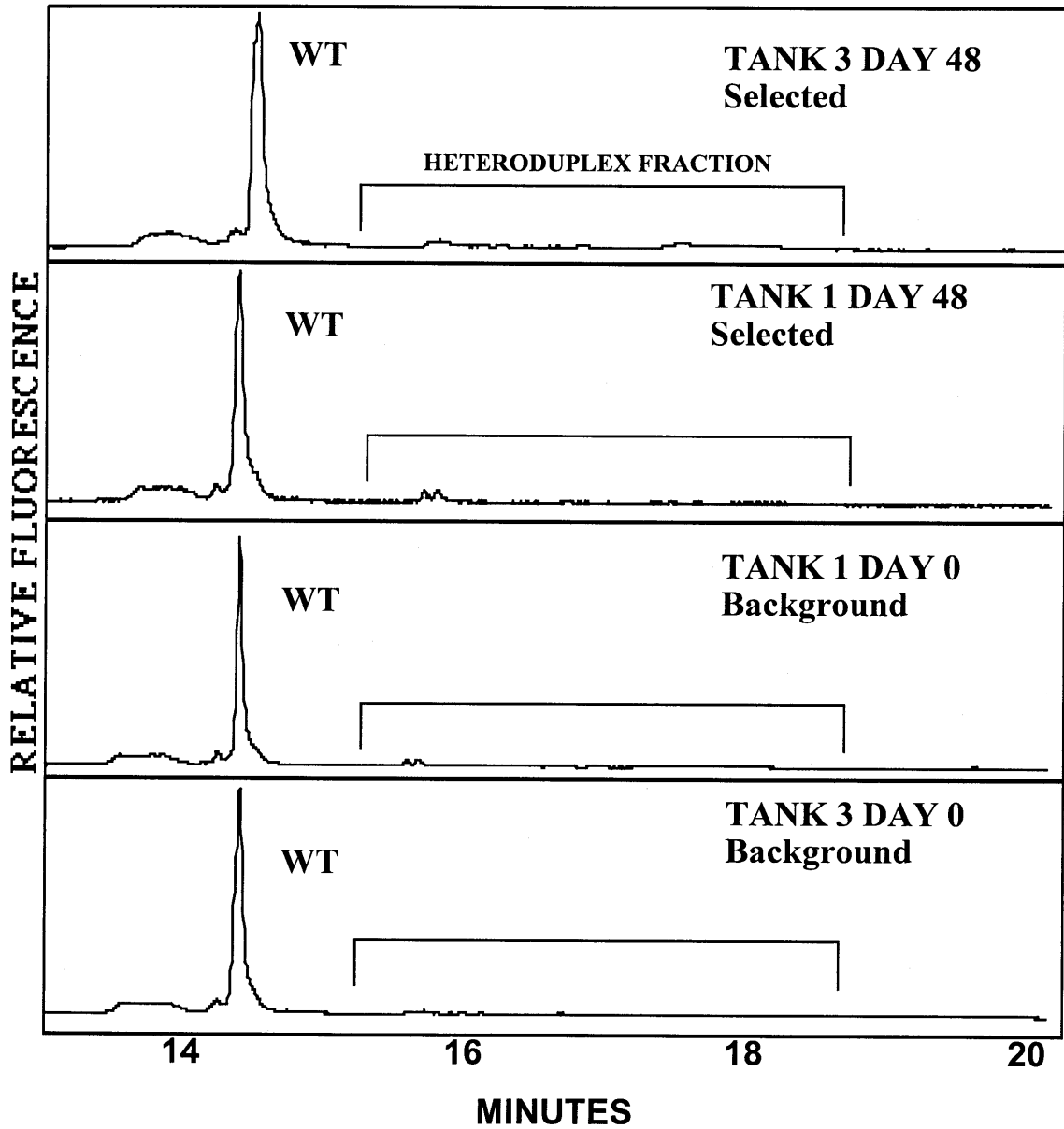
Figure 13: Collection for heteroduplex fraction for exon 2 with internal standard at mutant fraction of 10^{-2} (10x scale).
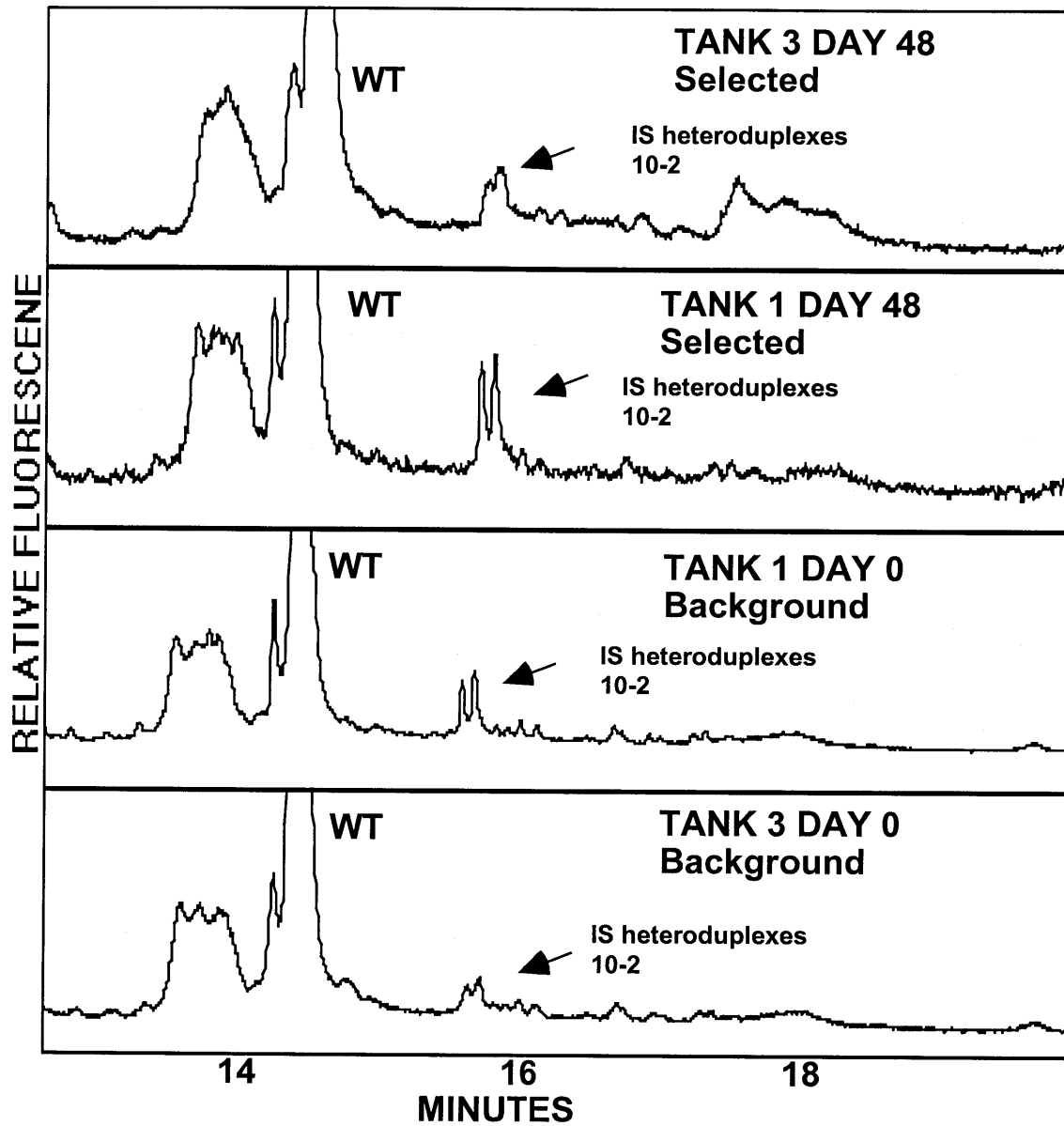
Figure 14: Demonstration of CDCE separation of wildtype and mutant homoduplexes for exon 2 after collection (68.8°C).
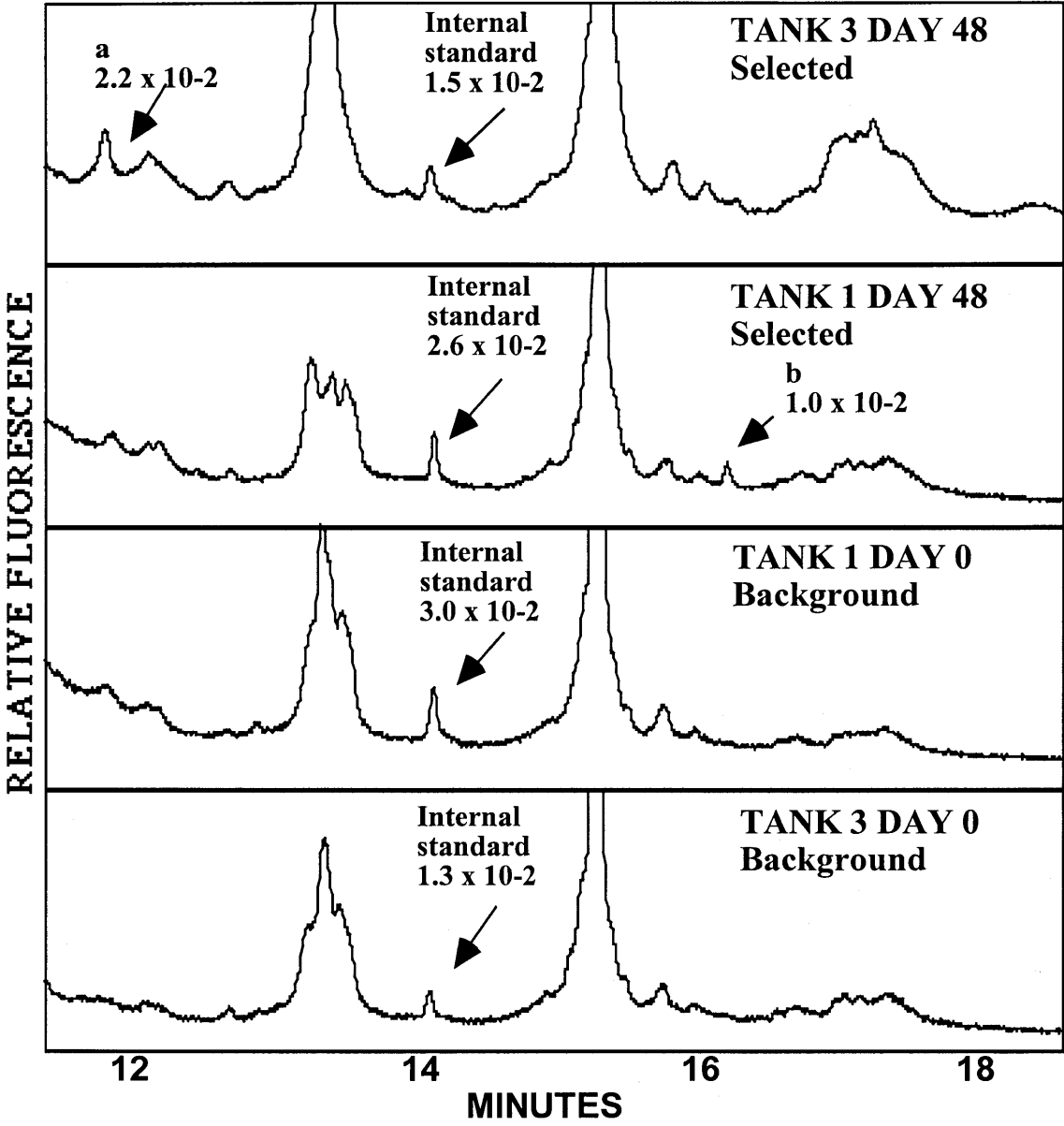
Figure 15: Demonstration of CDCE separation of wildtype and mutant homoduplexes including 6TGR day 0 sample after collection.
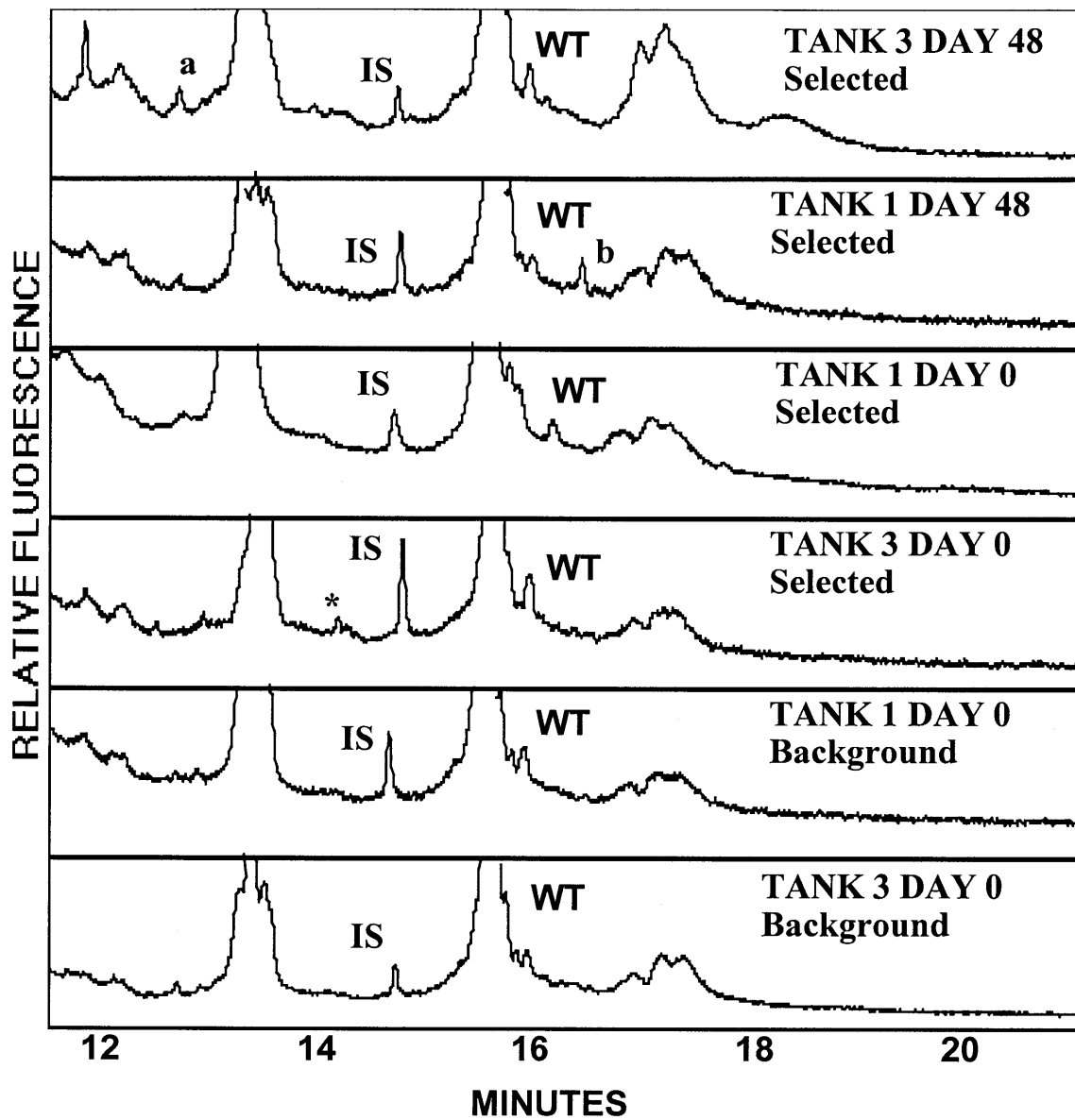
Figure 16: Spontaneous 6TGR mutations in exon 2

Figure 16 estimates the spontaneous point mutational rate for mutants a and b. The first column indicates the number of cells in culture. The second column indicates the expected mutant fraction after 60 doublings. The third column indicates the total of 6TGR mutants. The fourth column indicates the mutant fraction observed for exon 2 by CDCE. The fifth column indicates the calculated number of the mutant in question prior to 6TGR selection and accounts for the observation that 60% of spontaneously arising 6TGR mutants come from point mutations (Gennett and Thilly 1988). The last column is the calculated mutation rate for each particular mutant.

SPONTANEOUS 6TGR MUTATION IN EXON 2

MUTANT[1,2]	NO. OF CELLS	MUTANT FRACTION (60 DOUB.)	TOTAL NO. OF 6TGR MUTANTS ($\times 10^3$) (prior to selection)	%TOTAL 6TGR (observed)	NUMBER OF SPECIFIC MUTANT (prior to selection)	%HOTSPOT MUTATION RATE
a	2.4×10^9	1.2×10^{-5}	48	0.2	58	1.44×10^{-10}
b	2.4×10^9	1.2×10^{-5}	48	0.1	29	7.2×10^{-10}

[1] Mutant a observed in Tank 3 only.

[2] Mutant b observed in Tank 1 only.

4.2   Analysis of Exon 3, High Melting Region

The electropherograms of CDCE analysis in figure (17) demonstrates the heteroduplex separation from wildtype. The electropherogram includes a BPDE treated sample, which serves a positive control for detection of mutant in the exon 3 high melting region. A reproducible heteroduplex was present in both tank 1 and tank 3 at a mutant fraction of 0.5%.

Figure (18) indicates the heteroduplex region collected. An internal standard was spiked in at a mutant fraction of 10^{-2} in each of the samples.

Figure (19) demonstrates each sample as a homoduplex after a heteroduplex collection and high-fidelity PCR at separation temperature of 74.4°C. The internal standard clearly separated from the wildtype. A mutant was present reproducible in both tank 1 and tank 3. No peaks were present in tank 1 day 0 background, however peaks were present in tank 3 day 0 background at approximately the same elution time of the mutant in question.

Figure (20) demonstrates the same samples in figure 19 at a different separation temperature. However, again background noise from tank 3 day 0 eluted at approximately the same time as the mutant in question.

Figure 17: Demonstration of CDCE separation of heteroduplex region for exon 3 high melting region without internal standard.

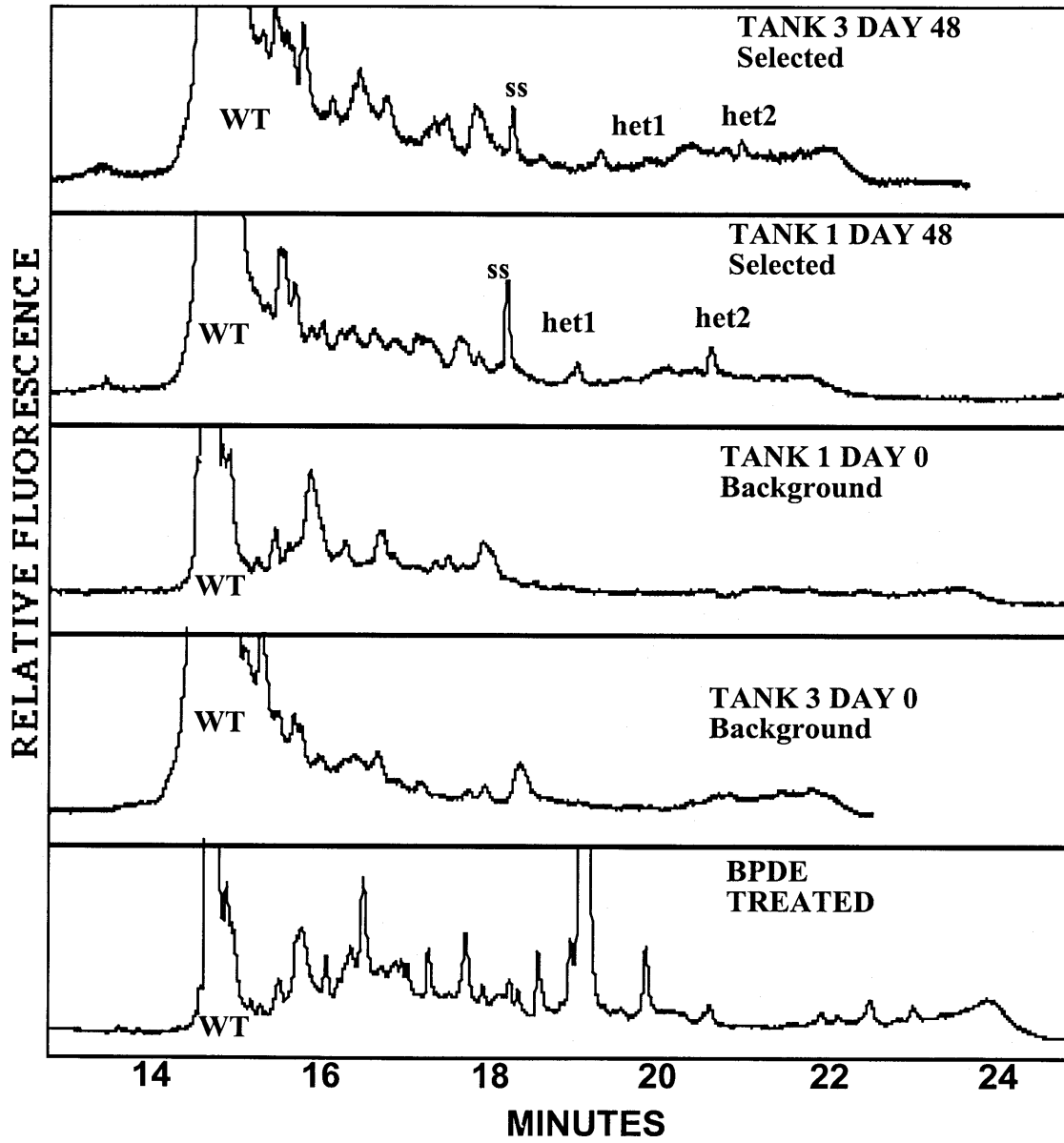Figure 18: Collection for heteroduplex fraction for exon 3 high melting region with internal standard at mutant fraction of 10^{-2} (Full scale).
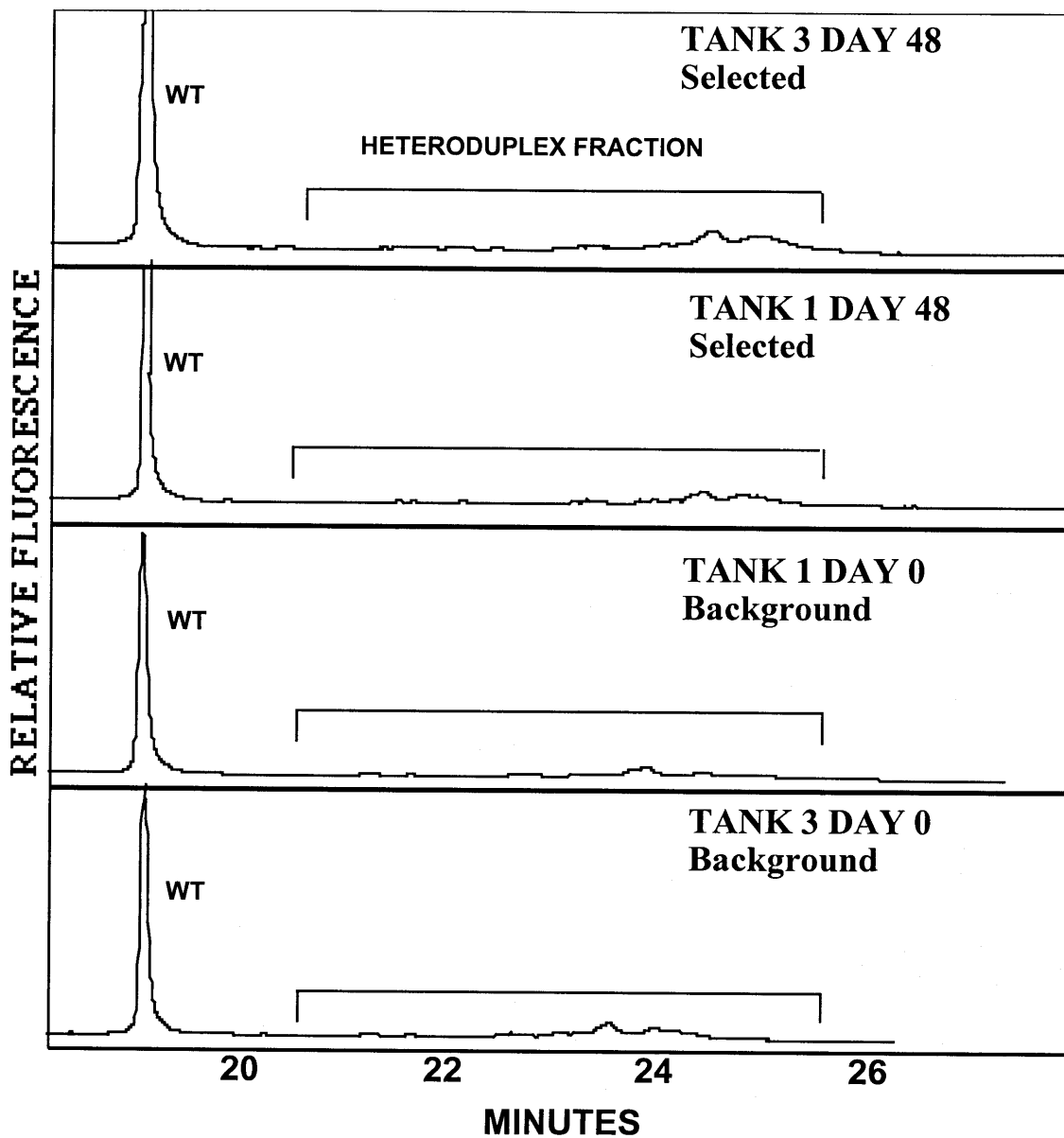
Figure 19: Demonstration of CDCE separation of wildtype and mutant homoduplexes for exon 3, high melting domain after collection (74.4°).
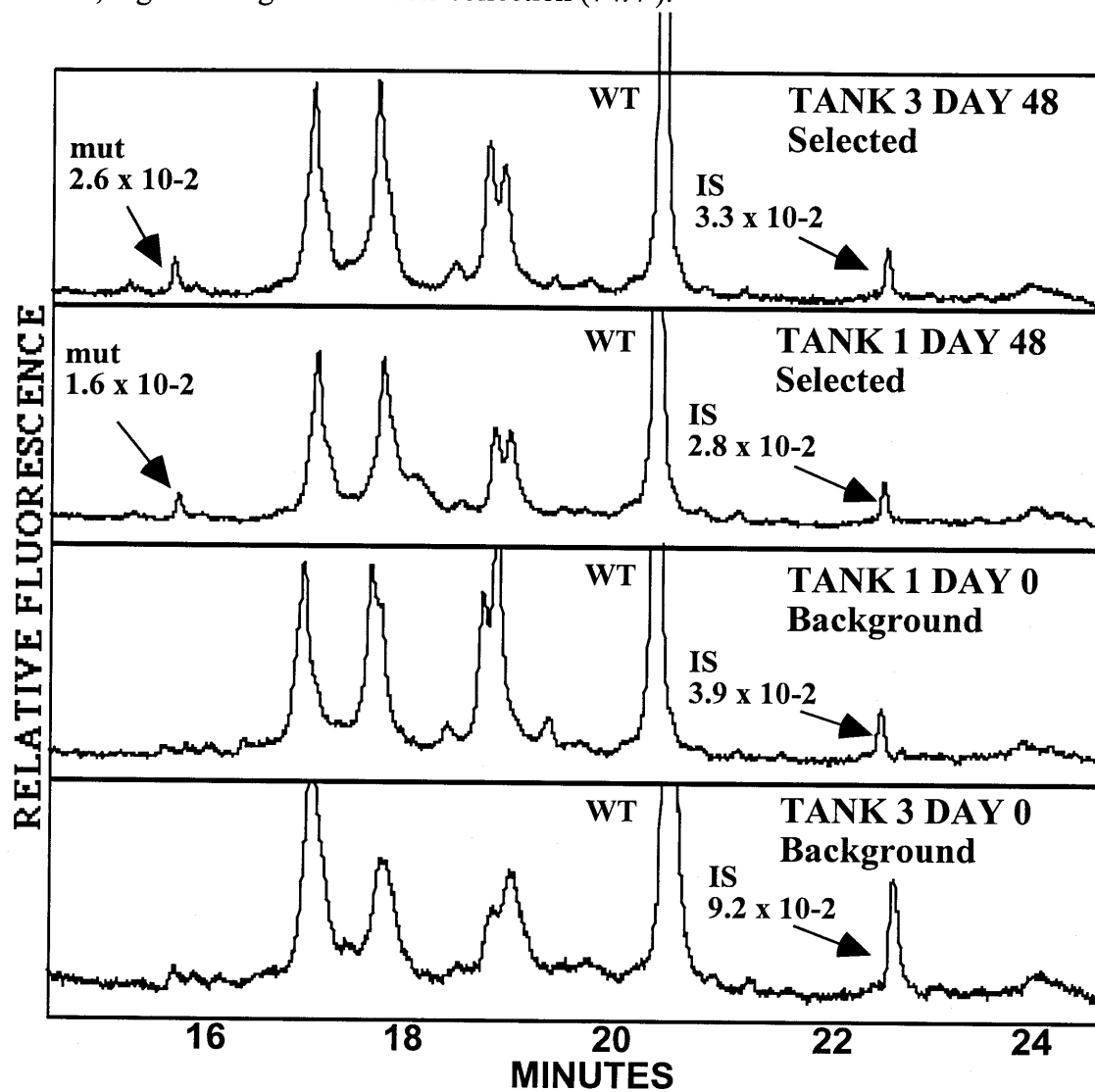
Figure 20: Demonstration of CDCE separation of wildtype and mutant homoduplexes for exon 3, high melting region after collection (74.8°).
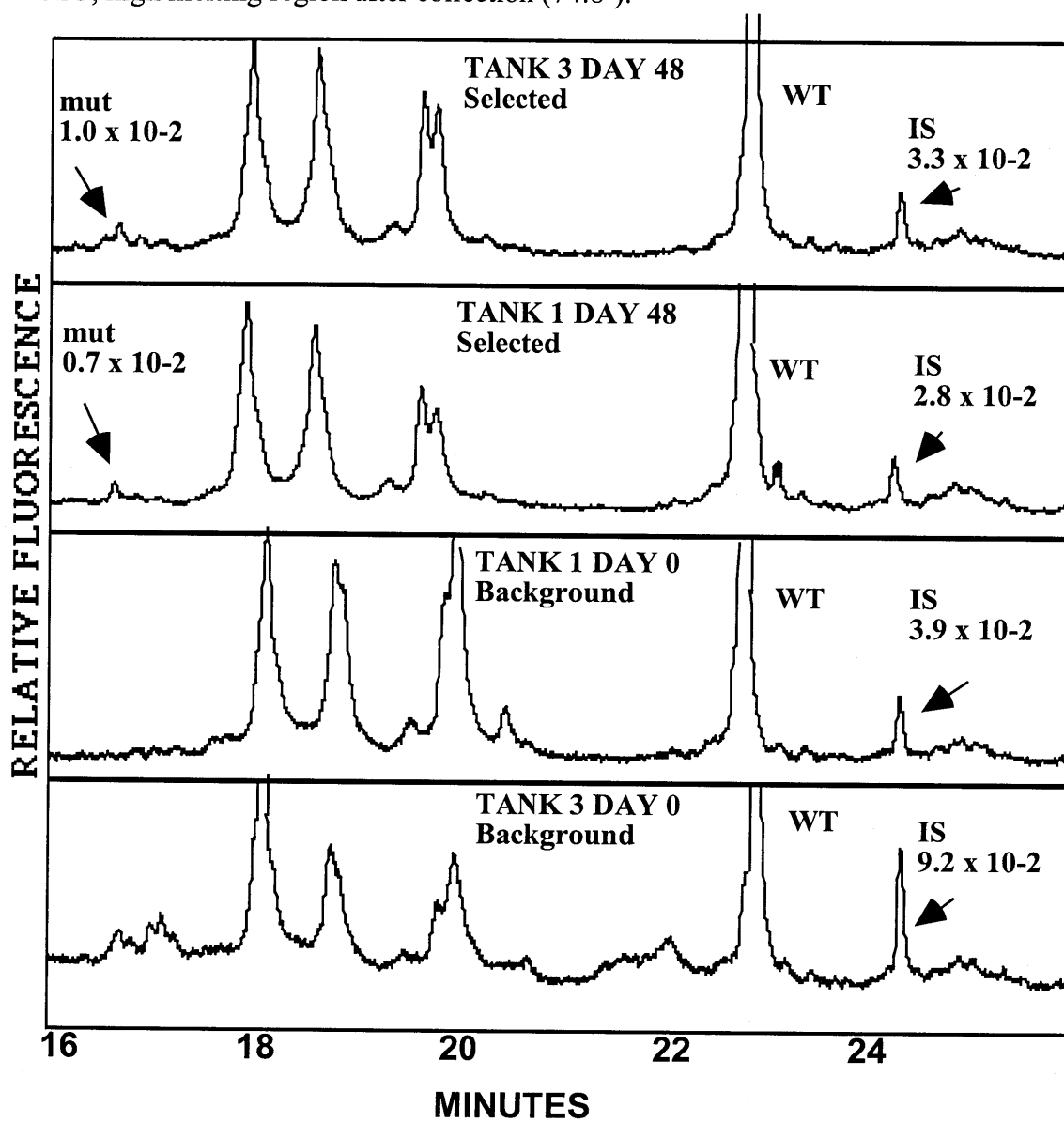
Figure 21: Spontaneous 6TGR mutations in exon 3, high melting region.

Figure 21 estimates the spontaneous point mutational rate for mutant X. The first column indicates the number of cells in culture. The second column indicates the expected mutant fraction after 60 doublings. The third column indicates the total of 6TGR mutants. The fourth column indicates the mutant fraction observed for exon 3 high melting region by CDCE. The fifth column indicates the calculated number of the mutant in question prior to 6TGR selection and accounts for the observation that 60% of spontaneously arising 6TGR mutants come from point mutations (Gennett and Thilly 1988). The last column is the calculated mutation rate for mutant X.

SPONTANEOUS 6TGR MUTATION IN THE HIGH MELTING REGION OF EXON 3

MUTANT	NO. OF CELLS	MUTANT FRACTION (60 DOUB.)	TOTAL NO. OF 6TGR MUTANTS (x 10^3) (prior to selection)	%TOTAL 6TGR (observed)	NUMBER OF SPECIFIC MUTANT (prior to selection)	%HOTSPOT MUTATION RATE
X	2.4×10^9	1.2×10^{-5}	48	0.5	144	3.6×10^{-10}

5.   Discussion

The in vivo database for both the germline and somatic HPRT mutants appear to indicate that mutations arising from endogenous mechanisms may have a significant role in HPRT mutagenesis. This thesis set out to explore the kinds and frequencies of mutations arising spontaneously in the exon 2 and exon 3 high melting regions of the HPRT gene.

Exon 2 and exon 3 high melting region were analyzed using CDCE and high-fidelity PCR after six-liters of human lymphoblastoid cells had under gone 60 doublings. Mutants were observed in exon 2, however, were not present in both cultures and were lower than the experimentally defined hotspot detection of 5 x 10^{-3}. The low mutant frequencies may explain why each mutant is present in only a single culture. A mutant is present at 0.5% in exon 3 high melting, bordering on an experimentally acceptable level and will be sequenced and identified.

The in vivo somatic data appears to have several hotspots in the high melting region of exon 3. However, the results from this thesis indicate that this region has no large hotspots arising from spontaneous pathways.

6.   References

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