

Sensitive and Quantitative Detection of Somatic Tumor Mutations by Hi-Res Melting™ on the LightScanner®

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ABSTRACT

We have used LCGreen® Plus Hi-Res Melting on the LightScanner instrument, a 96-well plate-based platform, as a high sensitivity screen of tumor DNA samples for sequence variants. Altogether, 76 whole amplicon scanning assays from 18 cancer genes were developed and used to survey approximately 600 primary tumors.

Common tumor mutations in several of the whole amplicon scanning assays, such as BRAF exon 15, KRAS exon 2, and PIK3CA exons 10 and 21, showed that the LightScanner assays were capable of detecting multiple mutations that displayed varying mutant tumor to wildtype allele ratios. Further characterization by cloning or digital PCR followed by resequencing methods revealed that some of the mutations were detected in samples that contained as low as 2-10% mutant allele fractions. Over 150 somatic missense or insertion/deletion coding mutations have been identified in this project.

Of interest, for certain tumor samples, some common silent SNPs in several of the cancer genes, such as EGFR, KIT and PDGFRA, displayed a variant Hi-Res Melting profile from the common homozygous and heterozygous alleles. Comparison of the tumor and normal sequence data revealed a loss of the ~ 1:1 heteroduplex peak in the tumor suggesting a copy number change at this locus in these tumors. This data supports a somatic copy number change that is possibly due to locus amplification given that the genes surveyed are oncogenes. This data also suggests that Hi-Res Melting will be able to detect somatic copy number change events due to loss of heterozygosity (LOH) or homozygous deletion mechanisms that affect tumor suppressor genes.

In addition to the whole amplicon scanning assays, secondary LunaProbe™ genotyping assays have been generated to quantify the ratio of one allele to another in simulated samples containing 100%, 95%, 90%, 75%, 50%, 25%, 10%, 5% and 0% of a given allele. Optimally, both scanning and genotyping assays can be combined; data is presented on one such dual readout assay.

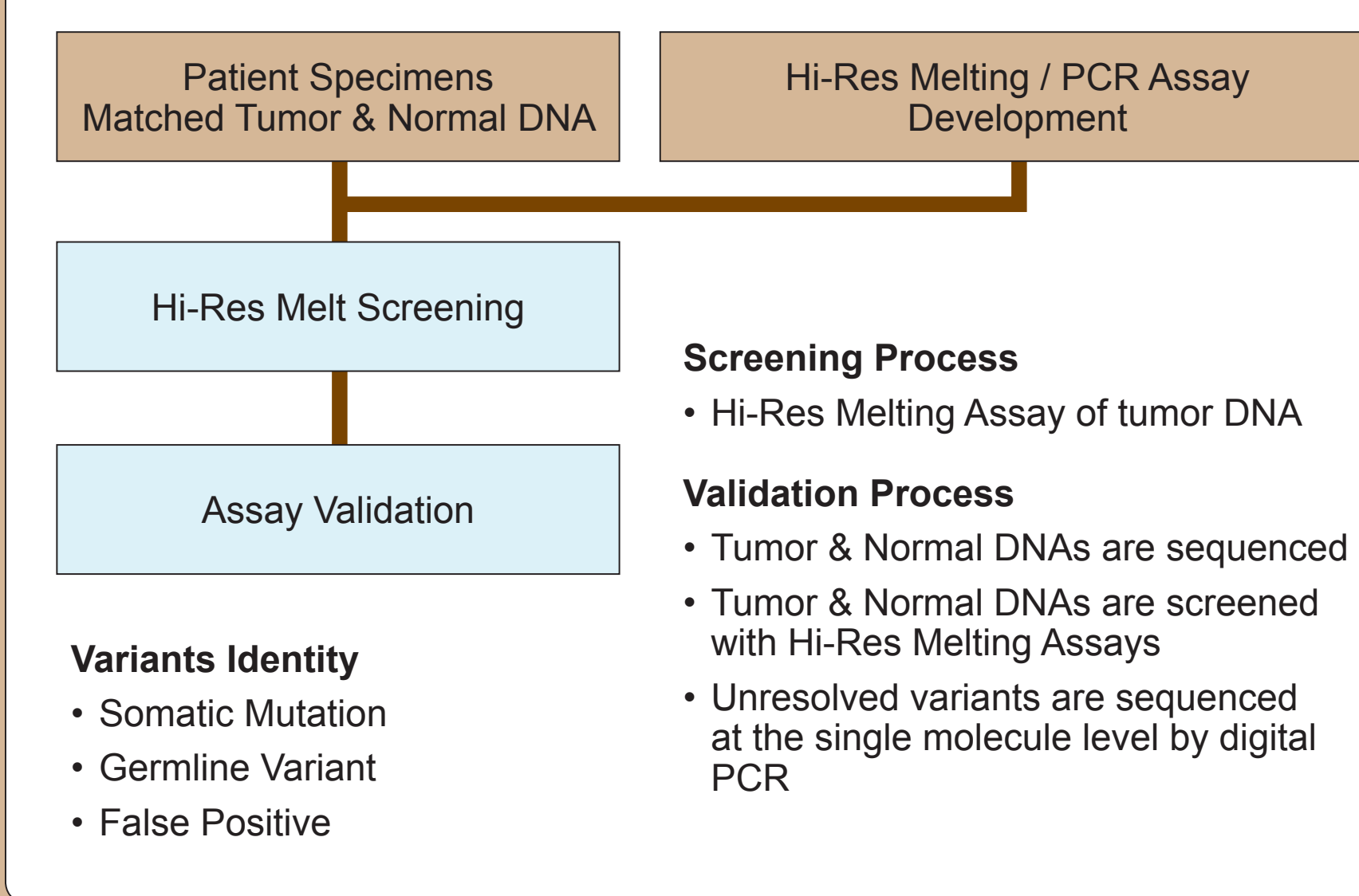
The combination of LightScanner scanning assays for discovery and LunaProbe assays for quantitative genotyping is attractive because this system is homogeneous, sensitive, specific, rapid, non-destructive, cost-effective and amenable to high throughput.

BACKGROUND

The discovery of somatic variants is vital for understanding the underlying mechanisms of tumorigenesis and potentially for patient selection. The primary technical challenge encountered in detecting somatic variants is the cellular heterogeneity that exists in tumor samples, e.g. a mutant allele may only be present at ~ 5-10% the level of the wild-type allele. Hence higher sensitivity assays are desired. Many methods have been utilized for the discovery of somatic mutations, but re-sequencing is still considered the gold standard. We have used Hi-Res Melting on the LightScanner® instrument, a 96-well plate-based platform, as a high sensitivity screen of tumor DNA samples for sequence variants.

The LightScanner platform was used to scan for variants in a diverse set of tumor types. Over 600 primary tumor samples were screened across 77 exons from cancer genes known to harbor somatic mutations including AXL, BRAF, EGFR, ERBB2, FGFR3, JAK1, JAK2, KIT, MET, PDGFRA, PDPK1, PIK3CA and RET.

Process Flow of Project



- Variants Identity**
- Somatic Mutation
 - Germline Variant
 - False Positive

Tumor DNA Collection

Tumor Tissue Types	601 Tumors
Bladder	42
Breast (IDC & ILC)	68
Carcinoid	8
Cervical	10
Colorectal	62
Endometrial (Adeno & Papillary)	11
Esophageal (Adeno & Squamous)	19
Head and Neck - Larynx & Oral	38
Hepatic (Cholangiocarcinoma & HCC)	14
Lung (NSCLC & Small Cell)	93
Melanoma	32
Ovarian	27
Pancreas	4
Prostate	38
Renal (Clear Cell & Papillary)	46
Sarcoma	27
Testicular (Embryonal & Seminoma)	19
Thyroid	43

RESULTS

LightScanner Hi-Res Melting assays for 77 exons from 18 cancer genes were used to screen ~ 600 primary tumor genomic DNAs. The amplicons for the Hi-Res Melting assays spanned the gene exon and flanking splice junctions, and ranged from 148 bp to 355 bp in size. Examples of 96-well plate melts are shown in Figures 1-5.

Figure 5 shows a common SNP in EGFR exon 20. This Q787Q SNP produced characteristic LightScanner melting profiles for the GG homozygote, the AA homozygote and GA heterozygote. The genotype of this SNP was confirmed by a LunaProbe genotyping assay (Fig. 6A). Of interest, unique variant melt profiles were distinguishable from those produced by the common SNP. These variant profiles were subsequently sequenced and shown to contain second sequence variant in addition to the Q787Q heterozygote (labeled H773H C/T + Q787Q G/A) or copy number variations of the silent Q787Q exonic SNP (e.g. B10 sample in Fig. 5).

After the initial rounds of screening, several assay variants yielded discordant calls between the LightScanner and sequencing results. The paired tumor and normal samples for all of these unresolved calls were analyzed by Hi-Res Melting with the relevant assays. A subset of these samples displayed tumor variant melt profiles compared to the matched normal DNAs—data for three of these samples are shown in Figure 7. Digital PCR was subsequently performed to identify the sequence variant and estimate the mutant allele fractions in those samples (expressed as a fraction of the number of diluted template wells that yielded mutant versus wildtype sequence).

Figure 1. LightScanner data for PIK3CA exon 21 (344 bp amplicon), Plate #3. Variants were detected in two head and neck tumor samples.

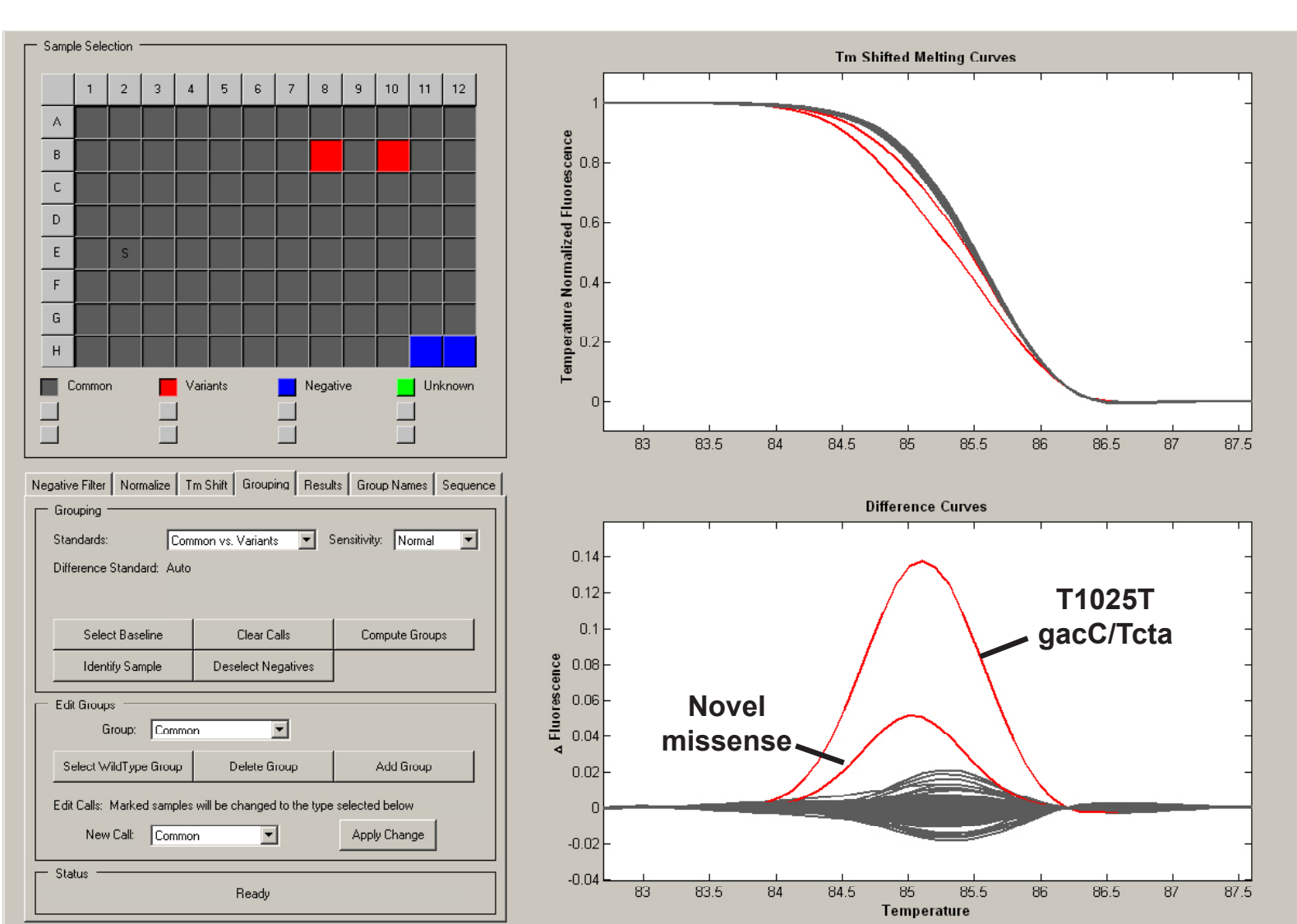


Figure 2. LightScanner data for PIK3CA exon 21, Plate #1. Mutations were detected in 12 breast cancer samples exhibiting varying mutant-to-normal allele fractions.

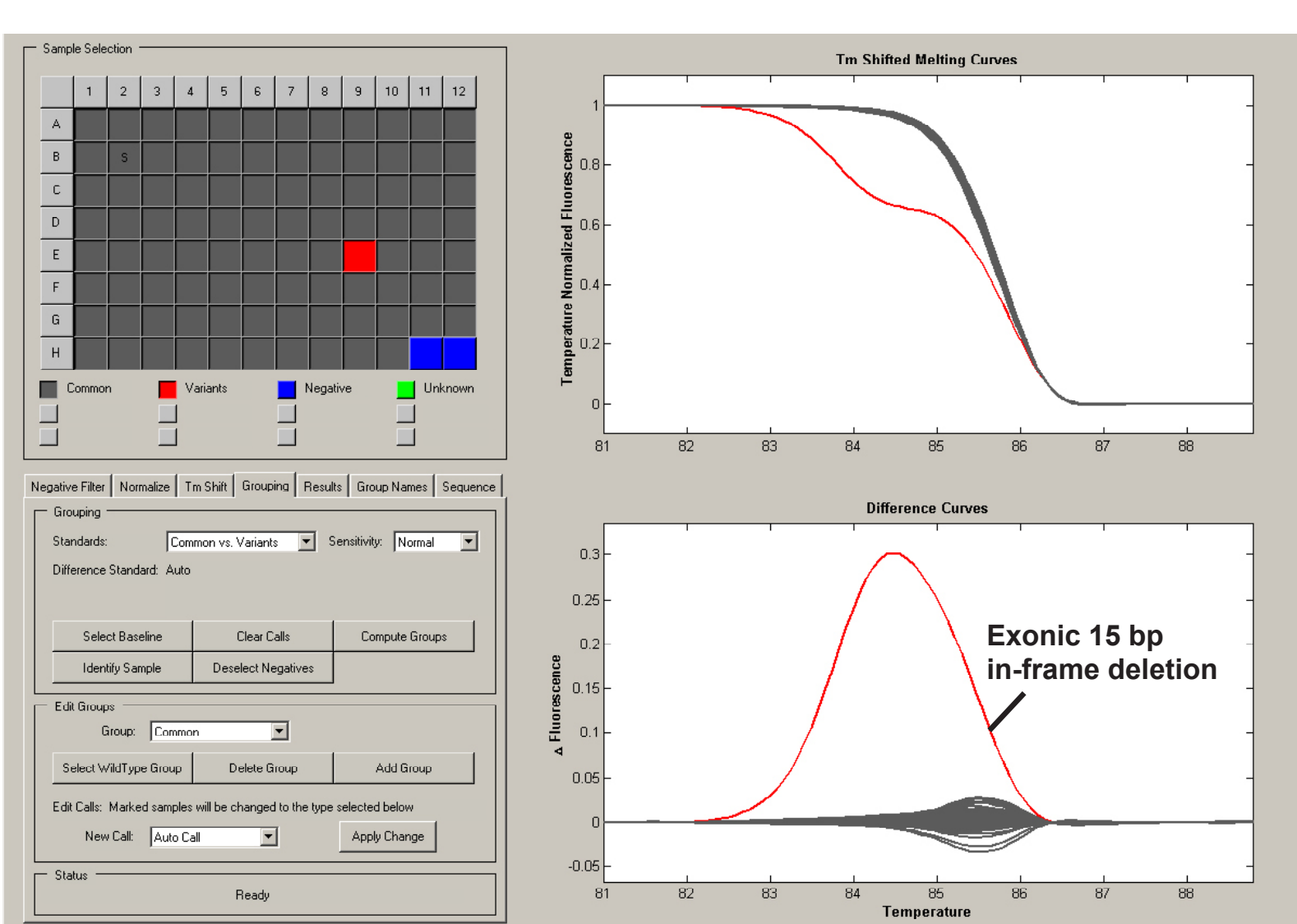


Figure 3. Data for EGFR exon 19, 148 bp amplicon. A mutation was detected in a non-small cell lung cancer sample.

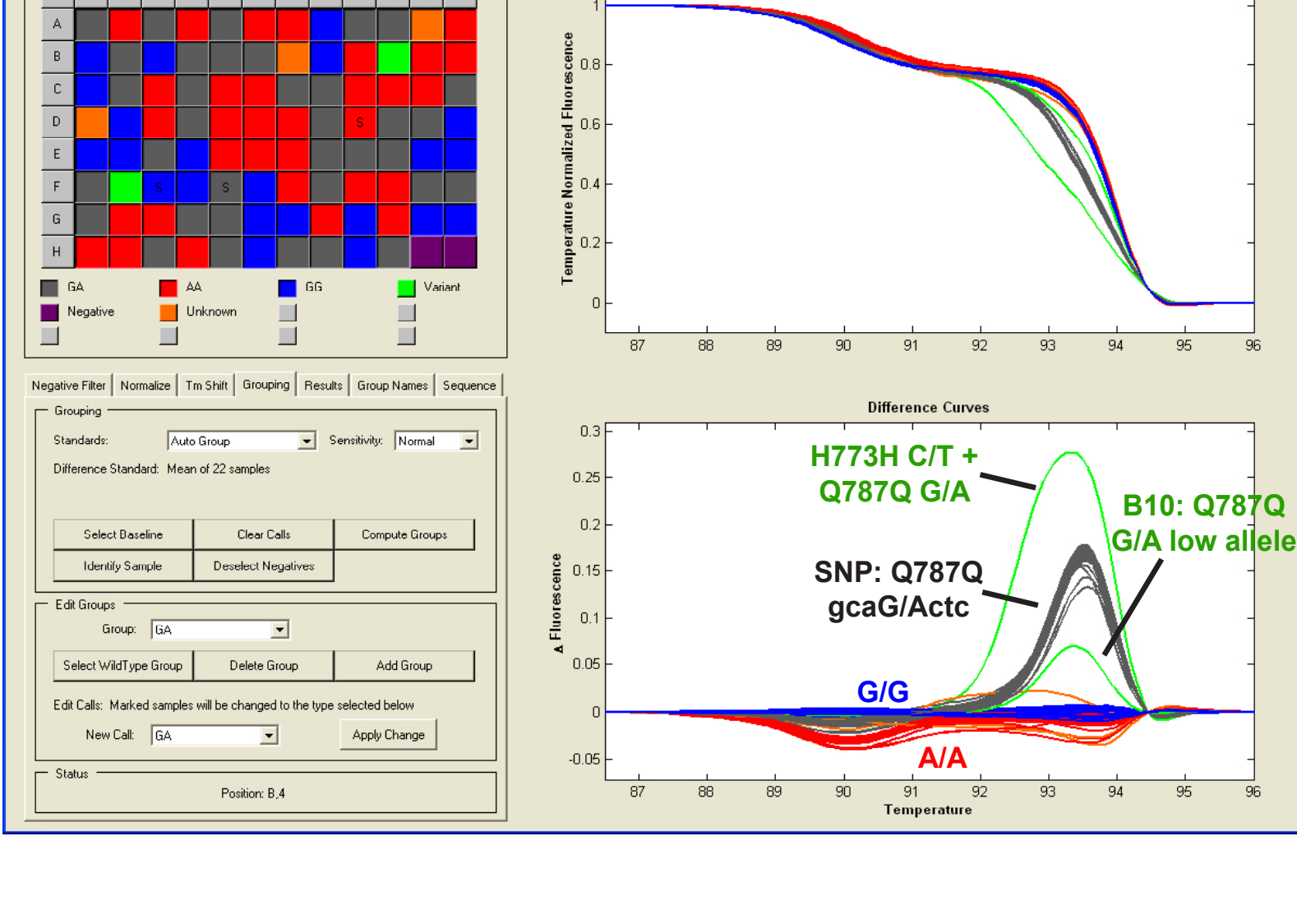


Figure 4. Data for EGFR exon 18, 213 bp amplicon.

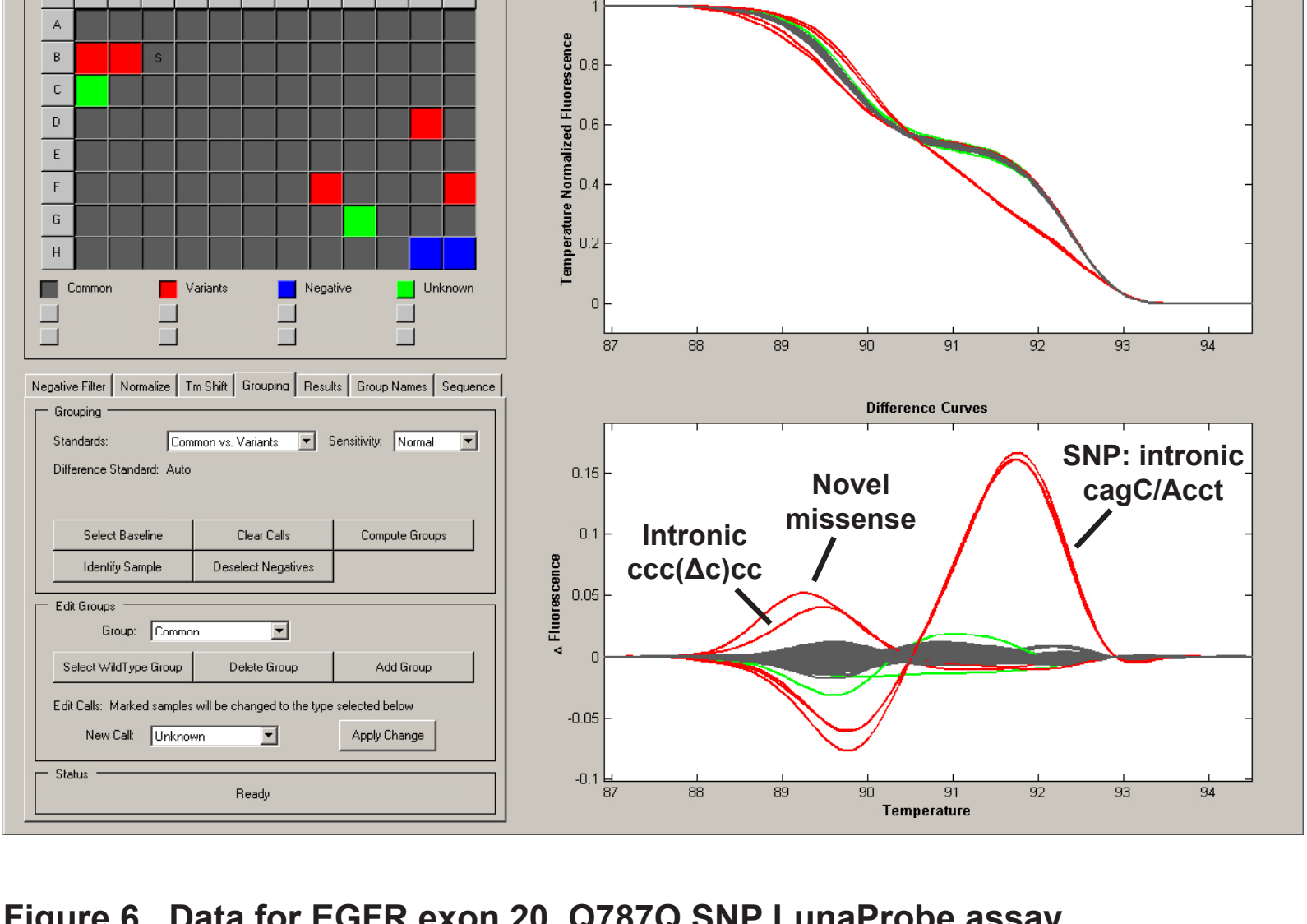


Figure 5. Data for EGFR exon 20 (248 bp amplicon). (A) Analyses of 96 primary tumor genomic DNAs. (B) Quantitative analyses of varying mixtures of homozygous A and homozygous G samples, along with heterozygous normal samples.

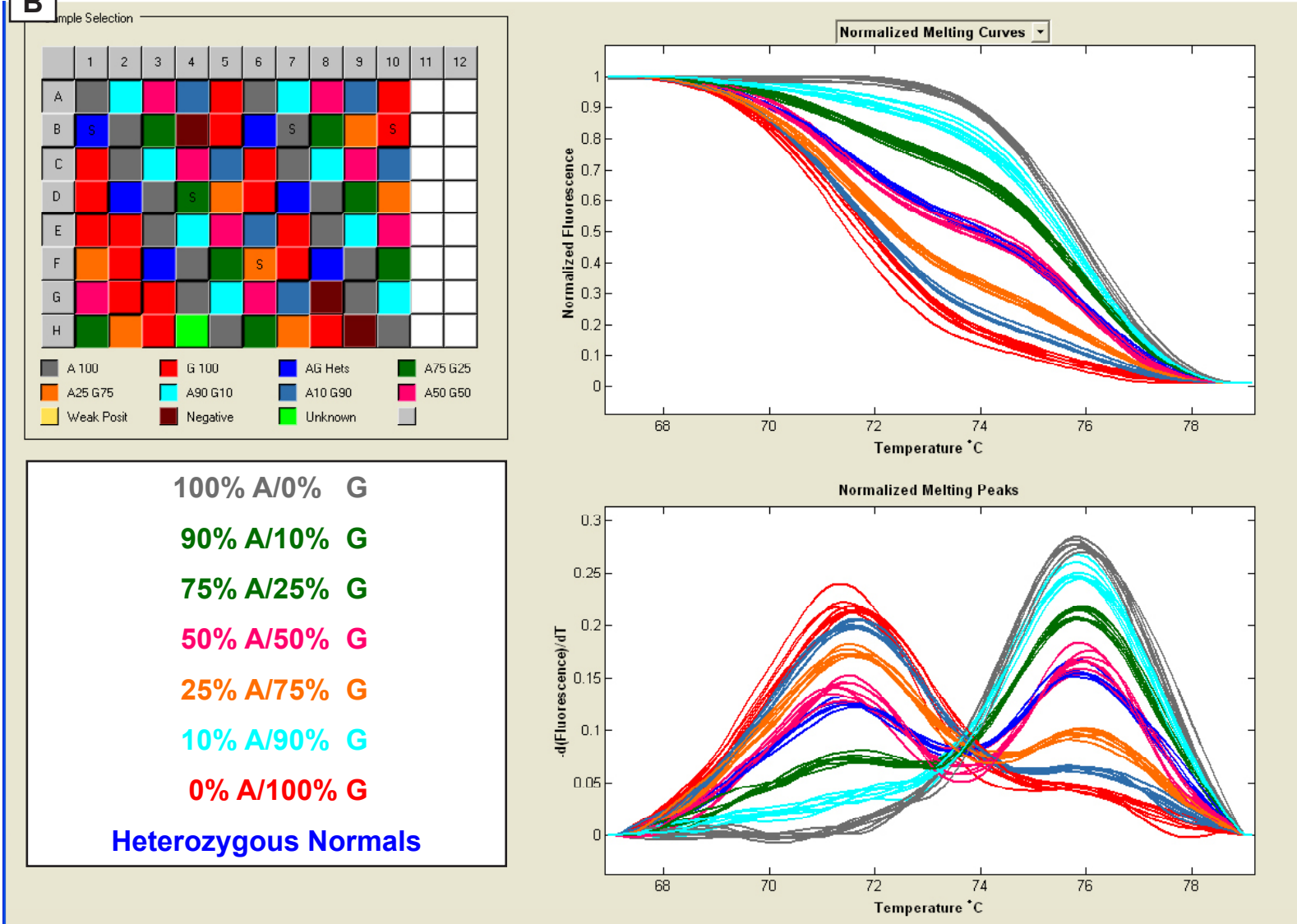
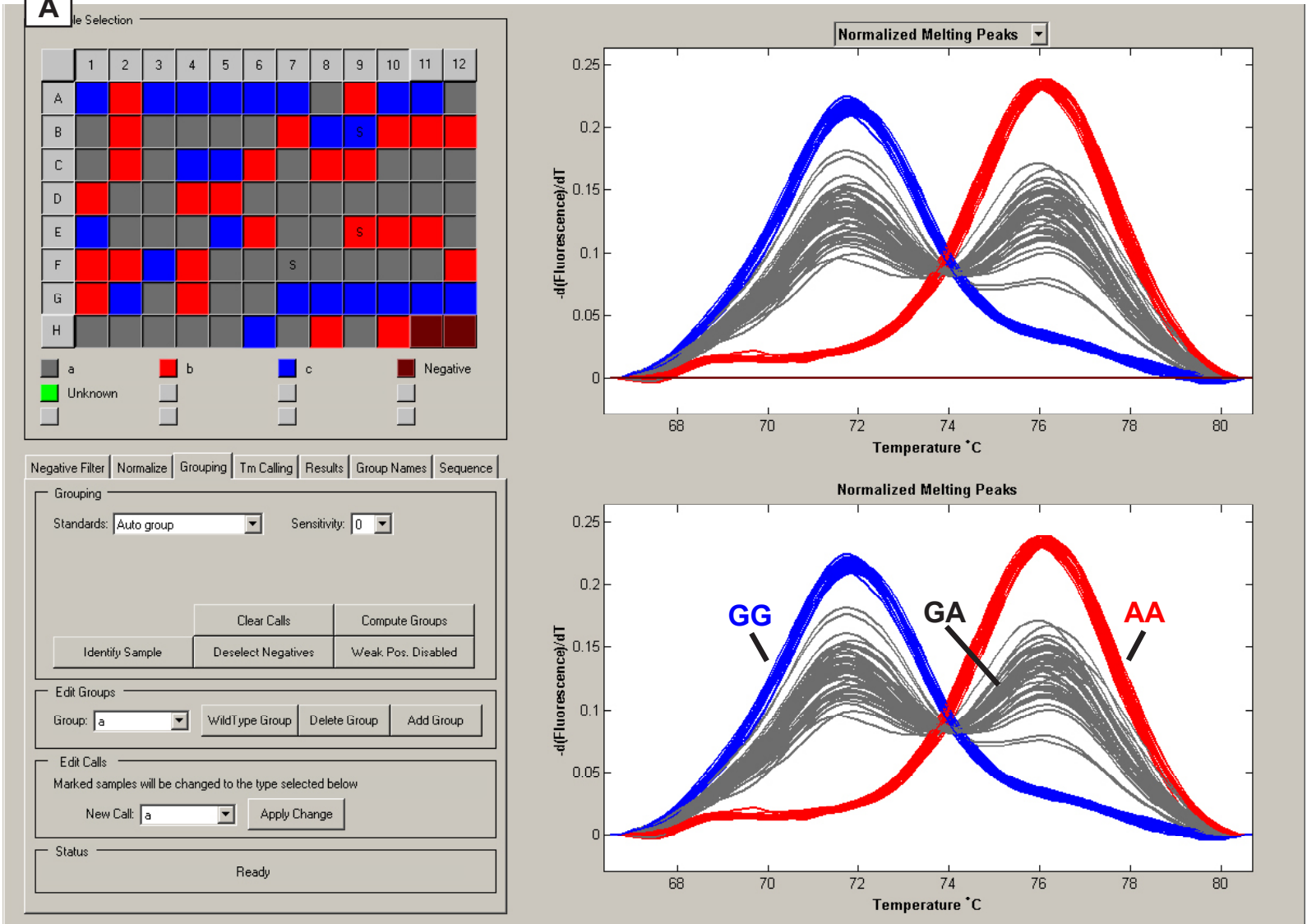
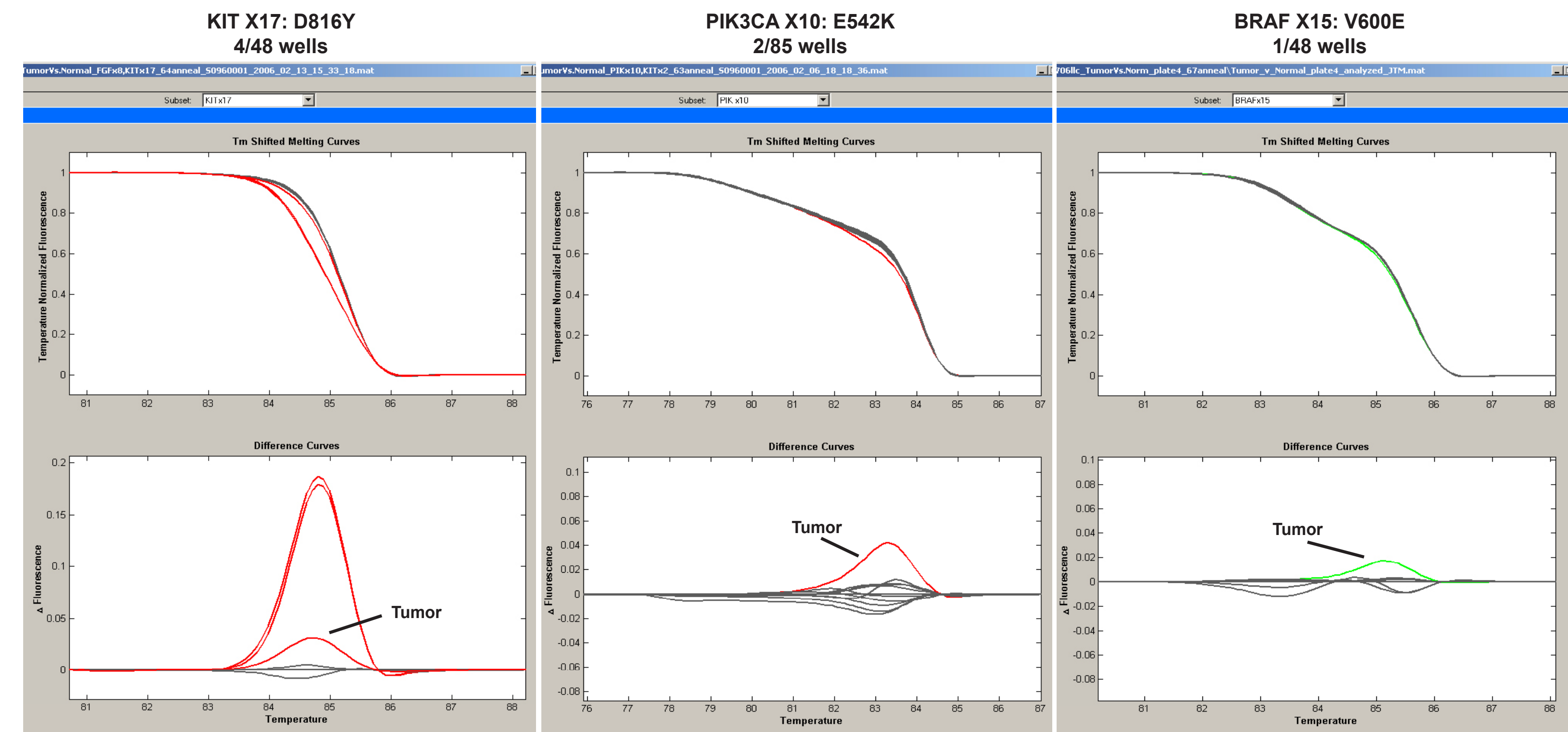


Figure 7. Paired tumor and normal data for three samples.



The sensitivity and specificity of Hi-Res Melting on the LightScanner was determined by a blinded re-sequencing study of 6 exons, 4 of which had a high prevalence of variants, using independently designed amplicons (Figure 8). A total of 695 amplicons were sequenced in both directions. The sequence data was assembled using both Phred/Phrap/Consed and Mutation Surveyor software packages. Two independent reviewers identified 90 variants that were detected by both packages. All 90 variants were detected by Hi-Res Melting, demonstrating 100% sensitivity. In addition to these 90 variants identified by re-sequencing, 25 more variants were detected by Hi-Res Melting. Eighteen of these variants were observed upon more comprehensive manual review of the sequence data. This indicates Hi-Res Melting was more sensitive than Sanger resequencing and performed with a specificity of at least 99%.

Figure 8. Summary of Sanger resequencing data used to determine the sensitivity and specificity performance of the LightScanner. Eight tumor DNA plates were analyzed using 6 different amplicons with multiple variants.

Assay	Plate	SBD* Somatic	SBD Germline	SBD Total	SBD Failures
BRAFx15	2	8	0	78	16
BRAFx15	3	10	0	79	15
BRAFx15	4	4	1	91	3
EGFRx20	3	0	34	91	3
FGFR3x6	1	4	21	91	3
KITx11	4	1	0	90	4
PIK3CAx10	2	0	0	90	4
PIK3CAx21	1	7	0	85	9
Totals	8	34	56	695	57

Initial Sequence Based Detection Variants* 90
VS
LightScanner Variant Calls 115

- All 90 SBD Variants called by LightScanner Melts
 - ➔ No False Negatives
- Secondary SBD Analysis: 18 additional variants detected
 - ➔ LightScanner sensitivity > Sequencing sensitivity by approx. 20%
- 7 LightScanner "False Positives"/587 SBD True Negatives
 - ➔ > 98.8% specificity

SUMMARY OF DATA

LightScanner

Total Hi-Res Melt Reactions	45,578
Variant Calls	799
Unknown Calls	310
Total "Variant + Unknown" Calls	1109
Sequencing Variants	965 (2.1%)
Germline	787 (1.7%)
Somatic	178 (0.4%)
Missense	147
Insertion/Deletion	8
Silent	17
Splice Site	2
Intronic	3

Δ144 (0.3%)

Overall, ~ 45,000 reactions were scanned and Hi-Res Melting detected over 900 variants including base (single and multiple) substitutions and insertions/deletions. The majority (~ 65%) of the variants detected were determined to be germline based on Sanger resequencing of both tumor & normal samples. The dominant type of somatic mutations were missense mutations (83%). Also found were splice site, in/dels, nonsense and silent changes. Based on resequencing data, the total Hi-Res Melting false positive rate was estimated to be around 0.3%.

This study demonstrates that Hi-Res Melting using the LightScanner instrument is a highly sensitive and specific, high throughput method that can be used to screen for somatic mutations in heterogeneous tumor samples.

CONCLUSIONS

- Simple, rapid, low cost and amenable to high throughput
- Closed (homogeneous) system minimizes amplicon contamination
- Non-destructive → used for resequencing downstream
- Over 600 primary tumors scanned for mutations
- Over 45,000 total reactions analyzed on the LightScanner
- Enabling software for automated variant calls and review
- > 75 LightScanner Hi-Res Melting assays developed for cancer genes; capable of genotyping
- 2 assays with high frequency exonic SNPs (EGFR exon 20 and FGFR3 exon 6) were successfully developed to detect additional variants
- Over 600 primary tumors scanned for mutations
- Over 45,000 total reactions analyzed on the LightScanner
- Highly sensitive (100%) and specific (99%) for heterozygote and somatic mutation detection, even in samples with low mutant allele fraction
- LunaProbe assays can quantify allele fractions