Abstract
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 heterogeneity that exists in tumor samples, e.g., a mutant allele may only be present at ~5-10% 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-throughput screen of tumor DNA samples for sequence variants.

The LightScanner platform was used to scan for variants in a diverse set of 10 tumor types. 376 tumors were tested across 289 regions from 9 genes known to harbor somatic mutations: Axl, B-Raf, EGFR, FGFR3, JAK2, MET, PIK3CA and RET. Overall, 10,246 reactions were scanned and Hi-Res Melting detected 215 variants including single (single) and multiple substitutions and deletions. Subsequent characterization of paired tumor and normal samples revealed that 97 of the 215 variants were only present in the tumor samples and were therefore somatic in origin.

The sensitivity and specificity of Hi-Res Melting was determined by a blinded re-sequencing study of 6 exons, 4 of which had a high prevalence of variants, using independently designed amplicons. 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 variants were detected by Hi-Res Melting. Eighteen of these variants were observed upon a more comprehensive manual review of sequence data. This indicates Hi-Res Melting was more sensitive than sequencing and performed with a specificity of at least 98.8%.

LightScanner Assay Development
Genomic DNA sequences for the human genes in this study were obtained from GenBank. Analysis was ex-locus based upon those coding regions where somatic mutations had been observed. Amplification primers were designed using LightScanner Primer Design Software. Forward and reverse primers have a minimum of 5 nucleotides of flanking intron sequence 5’ or 3’ of the coding region respectively. Candidate reagents were assessed by annealing temperature gradient PCR. Amplification reactions (10µl) were prepared using Idaho Technology 2.5X LightScanner Mastermixes that contain buffers, dNTPs (200 µM final concentration for each), 1X LCGreen Plus, MgCl₂ (2 mM final concentration), and a heat-start polymerase. Ten nanograms of human DNA is provided as amplification template and primers are included at 0.25 µM each. Cycling conditions are as follows: 1 minute at 94°C, followed by 40-45 cycles of (94°C, 30 seconds, and 62°C-72°C annealing temperature gradient, 30 seconds). If the region being amplified is of high GC content, DMSO, at a final concentration of 5%-10% volume to volume, is included. Products of the annealing temperature gradient PCR are assessed by gel separation on the LightScanner and by agarose gel electrophoresis. The annealing temperature provides an optimally robust, single-band product is selected for further development. To validate that the condition selected will provide robust, single band product, an assay is performed where 15 independent samples are assessed in duplicate. PCR reactions are prepared using LightScanner Mastermixes, 0.25 µM each primer, and 10 ng of template DNA as described above. Cycling conditions are as described above, except the annealing temperature is that which is selected and a final denaturation and re-annealing step (94°C, 30 seconds refer to Fig. 4) is added to the end of the cycling protocol. Denaturation and re-annealing is used to maximize heteroduplex formation should a heterozygous sequence variant be present. The plate of duplicate samples is scanned by melt profiling in the LightScanner. The reagents and the condition at which they function most effectively are considered validated if the melting profiles of duplicate samples are identical. DNA sequence analysis is then used to confirm the identity of the amplification product. Validated assays are used to assay the tumor DNA specimens provided by Novartis.

Results
LightScanner Hi-Res Melting assays for 28 exons from 9 cancer genes were used to screen 376 tumor genomic DNA. The amplicons for the Hi-Res Melting were designed to amplify the gene and flanking splice junctions, and ranged from 148 bp to 355 bp in size. Examples of 96-well plate melts are shown in Figures 2-7. The amplicons for the 2 of the 28 assay, EGFR X20 and FGFR3 X5, each contained a high frequency Specific Nucleotide Polymorphism (SNP). Figure 8 shows that for EGFR X20, the Q767R SNP produced characteristic LightScanner melting profiles for the GQ homozygote, the AA homozygote and GA heterozygote. The genotype of this SNP was confirmed by an independent SimpleProbe genotyping assay (Fig. 9). Internet, 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 Q767R heterozygote (labeled H7137 C/T = Q767R G/A) or varying allele fractions of a silent PCN3A heterozygote (e.g. 8110 sample with the Q7E8 allele shown in Fig. 8).

After the initial round of screening, there were 36 assay variants which 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. Five of these samples displayed tumor variant allele fractions in both LightScanner and sequencing results. The sequence data was subsequently 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 variants were detected by Hi-Res Melting. Eighteen of these variants were observed upon a more comprehensive manual review of sequence data. This indicates Hi-Res Melting was more sensitive than sequencing with a specificity of at least 98.8%.

Sequencing Identification of Variants
Independent PCR-based sequencing assays were designed and developed at Novartis. Standard Sanger sequencing reactions were performed and run on an ABI 3730xl DNA analyzer. The sequence data was subsequently assembled and analyzed using the default setting for both Phred/Phrap/Consed and Mutation Surveyor software packages. Two independent reviewers confirmed variants detected by both packages. When additional variants were detected in samples by Hi-Res Melting on the LightScanner, the sequence data was reviewed manually to identify low allele variants. In some of these cases, digital PCR was performed followed by downstream sequencing to confirm the presence of the variant. Paired tumor and normal samples were routinely analyzed to ascertain if a variant was somatic or germline in origin.

Summary of Data for 1st Quarter of Project

Conclusions
• Simple, rapid, low cost and high throughput
• Closed/homogeneous/lysimeter systems minimize amplification
• Non-destructive; used for nosing down downstream
• Do not require automated sample collection with manual options
• 28 LightScanner Hi-Res Melting assays developed, capable of genotyping
• 2 assays with high frequency exonic SNPs (EGFR exon 20 and FGFR3 exon 6) successfully developed to detect additional variants
• Processed 109 single-use tumor DNA plates, each containing 94 samples and 2 negative controls
• 10,264 total variants analyzed on the LightScanner
• Highly sensitive (100%) and specific (> 99%) for heterozygote detection, even in samples with low mutant allele fraction
• If required, samples can be spiked with wild type template for detecting homozygous variants (scanning)