Highly Sensitive Detection of EGFR T790M on Ion Torrent PGM

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Abstract #3490

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Introduction

EGFR T790M mutation leads to treatment resistance in ~50% of non-small cell lung carcinoma (NSCLC) patients undergoing tyrosine kinase inhibitor (TKI) treatment. Early detection of the emergence of this resistance mutation allows for tailoring of the treatment regimen. Detection of EGFR T790M can be useful for monitoring treatment resistance, so it is useful to have access to high sensitivity techniques to monitor the fluctuations in mutation frequency. (**refs 1, 2**). Here we demonstrate that our proprietary amplicon next-generation sequencing (NGS) assay on the Ion Torrent PGM provides an attractive option for EGFR T790M detection with the advantages of high sensitivity, specificity, and quantification capability.

Materials and Methods

We developed a proprietary library preparation method for amplicon sequencing on the Ion Torrent PGM. We employed this method (non-enrichment method) to detect and quantify the EGFR T790M mutation. To further enhance our sensitivity to detect low frequencies of T790M mutations, we developed a proprietary PCR-based method for enriching the T790M frequency before sequencing on the PGM (enrichment method). We only used a subset of the Ion Torrent barcodes since we found that several of them resulted in cross-contamination. Sequencing data were analyzed with Torrent Suite 2.2 (**ref 3**) and the BAM files were manually reviewed in Integrative Genome Viewer (IGV). This manual review was necessary for quantification of variants below 1%. The hg19 human genome was used as reference, and a portion of EGFR exon 20 was used as the region of interest. DNA from NCI-H1975 and NA19240 cell lines were used to determine the limit of detection (LOD), sensitivity, specificity, accuracy and reproducibility of our method. We also used reference FFPE DNA from HorizonDx to validate the accuracy and robustness of our assay. We further validated the test by comparing the performance of our NGS method with droplet digital PCR (ddPCR) using the same sample set (serially diluted H1975 into wild-type DNA controls).

Results

Testing model: In order to differentiate the PCR and sequencing errors from the true low-level T790M mutations, we established a cell line dilution model by taking advantage of a nearby *cis* mutation (Q787Q SNP) in the NCI-H1975 cell line, and the WT status of both positions in the NA19240 cell line. The Q787Q mutation allowed us to track whether a T790M mutation was a true positive or a false positive, since true positives would be expected to have both mutations and false positives would not (**Figure 1**). Variant frequencies of T790M and Q787Q in serially diluted samples showed *cis* mutation status and similar mutation frequencies (**Figure 2**), indicating the T790M detected is likely not from PCR error.

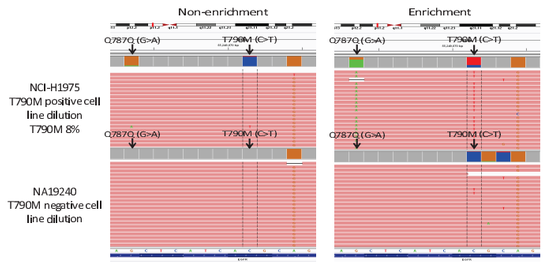

Figure 1: IGV visualization of the sequence reads for non-enrichment (left panel) and enrichment (right panel) methods using 8% T790M mutant (upper row) and WT NA19240 (lower row). The enrichment method shows the T790M mutation in both samples, but it can be identified as a false positive in the NA19240 sample since the Q787Q mutation is not observed. This false positive variant is due to the low level of background PCR error. A=green; C=blue; G=orange; T=red.

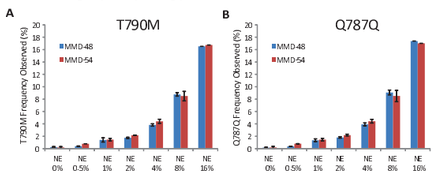

Figure 2: Histograms of observed mutation frequencies. Each dilution was run in duplicate in each of two sequencing runs. A. EGFR T790M mutation. B. EGFR Q787Q SNP. NE=Non-enrichment method. Graphs for enrichment method were also comparable (data not shown).

Principle of Determining LOD: The LOD was determined by the lowest percentage mutant that has minimum of 99% confidence to be non-wild type and is also in the linear range of the T790M quantification.

Analytical Sensitivity - Non-Enrichment Method: We used the non-enrichment method to sequence 23 DNA samples without the T790M variant (WT), and quantified the number of non-specific reads for T790M that were observed in each sample. These WT samples averaged 0.17% T790M reads (STD 0.085%) († in **Table 1**). We used the average proportion of non-specific reads plus 5x STD to determine a reasonable cut-off point to distinguish wild-type and mutant T790M samples in our non-enrichment method (0.6%). We used our non-enrichment method to sequence samples with a range of low frequency T790M variants (Table 2). Many of these samples were diluted at our facility, and others were purchased. Using just the variants with T790M frequencies greater than our cut-off of 0.6%, the linear regression comparing the expected and the observed frequencies generated an R2 = 0.9987 (**Figure 4A**), indicating the assay is quantitative. However, the quantification starts losing linearity at variant frequencies below 3.5% (**Figure 4B**).

Table 1: Sequencing of T790M WT samples to determine level of background variant calling (non-enrichment method).

Sample Name	Sample Type	% T790M Expected	Run Name	Barcode	% T790M Observed
NE NA19240 †	PCR	0	MMD-20	Bar1	0.09
NE NA19240†	PCR	0	MMD-20	Bar2	0.12
NE NA19240†	PCR	0	MMD-27	Bar4	0.13
NE NA19240†	genomic	0	MMD-31	Bar13	0.13
NE NA19240†	genomic	0	MMD-31	Bar14	0.13
NE NA19240†	genomic	0	MMD-31	Bar15	0.22
NE NA19240†	genomic	0	MMD-31	Bar16	0.13
NE NA19240†	genomic	0	MMD-48	Bar6	0.24
NE NA19240†	genomic	0	MMD-48	Bar7	0.23
NE NA19240†	genomic	0	MMD-48	Bar14	0.22
NE NA19240†	genomic	0	MMD-48	Bar15	0.4
NE NA19240†	genomic	0	MMD-48	Bar16	0.12
NE NA19240†	genomic	0	MMD-54	Bar6	0.16
NE NA19240†	genomic	0	MMD-54	Bar7	0.13
NE NA19240†	genomic	0	MMD-54	Bar14	0.16
NE NA19240†	genomic	0	MMD-54	Bar15	0.13
NE NA19240†	genomic	0	MMD-54	Bar16	0.43
NE NA19240†*	PCR	0	MMD-61	Bar13	0.14
NE NA19240†*	PCR	0	MMD-61	Bar14	0.13
NE NA19240†*	PCR	0	MMD-61	Bar16	0.14
NE 01*	genomic	0	MMD-69	Bar12	0.14
NE NA19240†*	genomic	0	MMD-69	Bar14	0.16
NE NA19240†*	genomic	0	MMD-69	Bar16	0.13

Note: The average frequency of T790M observed is 0.17% with STD 0.085%.
† Used original barcodes
* Used optimized barcodes

Table 2: T790M variants, ≥0.2% frequency (non-enrichment method).

% T790M	Sample Type	Run Name	Barcode	% T790M Observed	% Q787Q Observed
NE 0.33%H *	genomic	MMD-54	Bar12	0.78	0.8
NE 0.33%H *	genomic	MMD-54	Bar13	0.78	0.78
NE 0.35%H	genomic	MMD-31	Bar9	0.68	0.72
NE 0.70%H *	genomic	MMD-31	Bar8	0.82	0.83
NE 0.70%H *	PCR	MMD-20	Bar4	0.61	0.61
NE 0.70%H *	PCR	MMD-61	Bar3	0.85	0.84
NE 0.82%H *	genomic	MMD-54	Bar11	1.32	1.3
NE 0.82%H *	genomic	MMD-54	Bar10	1.6	1.6
NE 0.82%H *	genomic	MMD-48	Bar10	1.65	1.51
NE 0.82%H *	genomic	MMD-48	Bar11	1.17	1.2
NE 1.40%H *	genomic	MMD-31	Bar5	1.74	1.78
NE 1.40%H *	genomic	MMD-31	Bar4	2.5	2.4
NE 1.65%H	genomic	MMD-48	Bar9	1.67	1.72
NE 1.65%H	genomic	MMD-54	Bar9	2.18	2.28
NE 1.65%H *	genomic	MMD-48	Bar8	1.82	1.89
NE 1.65%H *	genomic	MMD-54	Bar8	2.15	2.1
NE 1.75%H *	PCR	MMD-61	Bar2	1.82	1.98
NE 3.29%H *	genomic	MMD-48	Bar4	3.7	3.75
NE 3.29%H *	genomic	MMD-48	Bar5	3.95	4.09
NE 3.29%H *	genomic	MMD-54	Bar5	4.18	4.21
NE 3.29%H *	genomic	MMD-54	Bar4	4.65	4.64
NE 3.50%H *	genomic	MMD-31	Bar2	4.58	4.75
NE 3.50%H *	genomic	MMD-31	Bar3	5	5.02
NE 3.50%H *	PCR	MMD-20	Bar3	3.36	3.46
NE 3.50%H *	PCR	MMD-61	Bar1	3.66	3.83
NE 7.00%H *	genomic	MMD-31	Bar1	7.1	7.26
NE 8.23%H *	genomic	MMD-54	Bar3	7.94	7.87
NE 8.23%H *	genomic	MMD-48	Bar3	8.57	8.79
NE 8.23%H *	genomic	MMD-48	Bar2	8.94	9.34
NE 8.23%H *	genomic	MMD-54	Bar2	9.03	9.15
NE 16.45%H *	genomic	MMD-48	Bar1	16.53	17.39
NE 16.45%H *	genomic	MMD-54	Bar1	16.74	17.02
NE 5 (0.15%) *	genomic	MMD-69	Bar8	0.28	50.72
NE 10 (0.28%) *	genomic	MMD-69	Bar7	0.46	50.89
NE 50 (1.28%) *	genomic	MMD-69	Bar5	1.72	51.35
NE 100 (3.06%)*	genomic	MMD-69	Bar4	3.32	53.01
NE 500 (13.91%) *	genomic	MMD-69	Bar3	14.34	56.97
NE 1,000 (24.26%)*	genomic	MMD-69	Bar2	25.44	60.01
NE 5,000 (73.09%) *	genomic	MMD-69	Bar1	71.7	76.23
NE Horizon Dx (6.5%) *	genomic	MMD-69	Bar13	6.86	0.34

* Used optimized barcodes

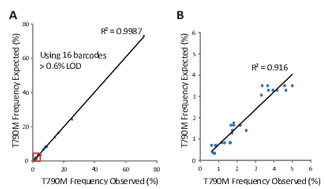

Figure 4: Observed versus expected T790M frequencies using the non-enrichment method and 16 barcodes . A. Plot of all data points. B. Detail plot of data points for low frequencies (≤ 3.5%), red boxed area in A.

Improvement of Non-Enrichment Method LOD Using 13 Optimized Barcode Set: Based on several sequencing runs, we concluded that some barcodes showed evidence of cross-contamination. Thus, we only used 13 of the 16 barcodes in subsequent sequencing analyses ("optimized barcodes"). Of the 23 DNA samples without the T790M variant (WT) that were sequenced earlier, we analyzed six of the samples that were labeled using just the barcodes that did not show evidence of cross-contamination (starred in **Table 1**). These WT samples averaged just 0.14% T790M reads (STD 0.01%). We used the average proportion of non-specific reads plus 6x STD to revise our cutoff point to distinguish wild-type and mutant T790M samples in our non-enrichment method (0.2%). We also reanalysed the data in **Table 2**, using a new cut-off of 0.2% T790M and using only the samples that did not show contamination (starred in **Table 2**). This revised dataset showed an $R^2 = 0.9995$ for a linear regression for samples with T790M frequencies between 0.2%-75% (**Figure 5A**), indicating the assay is quantitative. Furthermore, the R^2 for detection of the lowest frequencies of T790M (0.2-3.5%) showed a marked improvement in linearity (**Figure 5B**), as compared to when all 16 barcodes were used.

Analytical Sensitivity - Enrichment Method: We sequenced 13 wild-type DNA samples to quantify the T790M background noise, using the enrichment method with the optimized 13 barcodes. These WT samples averaged 5.24% T790M reads (STD 0.5%) (**Table 3**). We used the average proportion of non-specific reads plus 3x STD to determine a reasonable cut-off point to distinguish wild-type and mutant T790M samples in our enrichment method (6.8%). As expected, all samples showed a substantial increase in T790M frequencies after undergoing enrichment (**Table 4**). We were able to observe the presence of the T790M mutation in samples with an input DNA frequency of as low as 0.03% T790M (**Figure 6A**), which is enriched to ~6.8% frequency in this method. The comparison of the input and enriched frequencies remains linear at the low range of T790M frequencies (0.03 to 0.7%), with an $R^2 = 0.9967$ (**Figure 6B**). The cell line dilutions were made using two different WT lines, which had different frequencies for the Q787Q variant.

Table 3: Sequencing of T790M WT samples to determine level of background variant calling (enrichment method).

Sample Name	Sample Type	% T790M Expected	Run Name	Barcode	% T790M Observed	% Q787Q Observed
E NA19240	PCR	0	MMD-21	Bar1	5.53	0.71
E NA19240	PCR	0	MMD-21	Bar2	5.49	0.79
E NA19240	PCR	0	MMD-23	Bar1	4.93	0.08
E NA19240	PCR	0	MMD-23	Bar2	4.84	0.09
E NA19240	PCR	0	MMD-23	Bar3	4.88	0.1
E NA19240	PCR	0	MMD-23	Bar4	4.78	0.08
E NA19240	PCR	0	MMD-26	Bar4	4.49	0.17
E NA19240	PCR	0	MMD-62	Bar13	5.14	1.33
E NA19240	PCR	0	MMD-62	Bar14	6.07	1.51
E NA19240	PCR	0	MMD-62	Bar16	4.95	1.32
E 0	genomic	0	MMD-70	Bar12	6.06	51.52
E NA19240	genomic	0	MMD-70	Bar14	5.68	1.7
E NA19240	genomic	0	MMD-70	Bar16	5.18	2.4

Note: The average frequency of T790M observed is 5.23% with STD 0.5%.

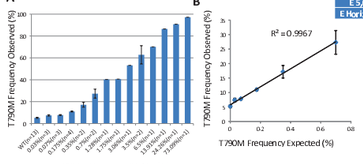

Figure 6: Observed versus expected T790M frequencies using the enrichment method and 13 optimized barcodes. A. Histogram including of all data points, B. Detailed plot of data points for low frequencies (0 to 0.7%).

Analytical Accuracy – Non-Enrichment Method: Three types of DNA (PCR products, genomic DNA and commercial FFPE genomic DNA) were used to assess the accuracy of the non-enrichment method. Two reference methods were used: Qubit-measured PCR mixture with accurately quantified mutation frequency, and droplet digital PCR (ddPCR).

PCR products: We PCR amplified NCI-H1975 and NA19240, and quantified the results by Qubit. We mixed the PCR products to generate samples with a range of known variant frequencies. The Ion Torrent platform was used to determine the frequencies of EGFR T790M and Q787Q (which is on the same allele). When the expected and observed mutation frequencies were compared, both mutations showed an $R^2 > 0.995$ (**Figure 7A** and **7B**).

Genomic DNA: We compared our non-enrichment method with our T790M ddPCR assay (**AACR 2013 Poster 3491**). Results obtained on the two platforms are in concordance with $R^2 > 0.9995$ (**Table 5** and **Figure 8**).

FFPE: We further analyzed the accuracy of the non-enrichment method by using reference FFPE genomic DNA containing the T790M mutation purchased from Horizon Diagnostics. The average T790M frequency determined by our method was 6.68% with STD 0.05%, concordant with the 6.5% frequency reported by Horizon Diagnostic using ddPCR (data not shown).

Table 4: T790M variants, ≥6.8% frequency (enrichment method).

% T790M	Sample Type	% T790M Expected	Run Name	Barcode	% T790M Observed	% Q787Q Observed
E 0.035%H	PCR	0.035	MMD-62	Bar11	7.29	2.95
E 0.035%H	PCR	0.035	MMD-62	Bar12	6.8	2.91
E 0.07%H	PCR	0.07	MMD-26	Bar3	7.89	3.62
E 0.07%H	PCR	0.07	MMD-62	Bar8	7.35	3.91
E 0.07%H	PCR	0.07	MMD-62	Bar10	8.09	4.28
E 0.175%H	PCR	0.175	MMD-26	Bar2	11.38	7.96
E 0.175%H	PCR	0.175	MMD-62	Bar5	10.89	7.94
E 0.175%H	PCR	0.175	MMD-62	Bar7	11.25	7.57
E 0.35%H	PCR	0.35	MMD-26	Bar1	18.7	15.49
E 0.35%H	PCR	0.35	MMD-62	Bar4	15.63	12.8
E 0.7%H	PCR	0.7	MMD-21	Bar4	30.21	27.1
E 0.7%H	PCR	0.7	MMD-62	Bar3	24.42	22.62
E 1.75%H	PCR	1.75	MMD-62	Bar2	40.61	40.55
E 3.5%H	PCR	3.5	MMD-21	Bar3	68.72	69.16
E 3.5%H	PCR	3.5	MMD-62	Bar1	56.9	58.14
E 1	genomic	0.03	MMD-70	Bar10	8.05	54.68
E 5	genomic	0.15	MMD-70	Bar8	10.46	55.6
E 50	genomic	1.28	MMD-70	Bar5	40.27	70.36
E 100	genomic	3.06	MMD-70	Bar4	53.18	75.23
E 500	genomic	13.91	MMD-70	Bar3	86.42	92.74
E 1,000	genomic	24.26	MMD-70	Bar2	90.78	95
E 5,000	genomic	73.09	MMD-70	Bar1	97.15	98.18
E HorizonDx	genomic	6.5	MMD-70	Bar13	70.11	1.6

Figure 7: Accuracy Analysis. Observed versus expected variant frequencies using the non-enrichment method, The samples were created by mixing genomic DNA of NCI-H1975 into NA19240. A. EGFR T790M mutation. B, EGFR Q787Q SNP.

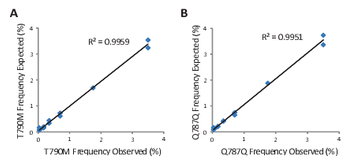

Table 5: Comparison of EGFR T790M mutation frequencies by ddPCR and NGS methods.

Labeled copy number	ddPCR % Observed	NGS % Observed
5,000	73.09	71.56
1,000	24.26	25.30
500	13.91	14.20
100	3.06	3.18
50	1.28	1.58
10	0.28	0.32
5	0.15	0.14
1	NA	0.04
0.5	NA	NA
0	0	0

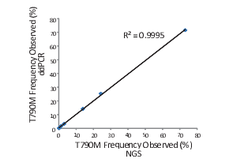

Figure 8: Comparison of EGFR T790M mutation frequencies observed by NGS non-enrichment method (x-axis) and ddPCR (y-axis) method.

Analytical Precision: The analytical precision, or coefficient of variation (CV), was calculated based on the 8 independent experiments that were run using the 13 optimized barcodes for both non-enrichment and enrichment methods (**Tables 6 and 7**). The T790M frequency was reproducibly measured by both the non-enrichment method and the enrichment method (LOD: 0.2% for non-enrichment and 6.8% for enrichment method).

Table 6: Analytical precision (CV) of non-enrichment method.

% T790M	Replicates	Average	STD	CV
NE 0%	7	0.14	0.010	0.071
NE 0.03%	3	0.22	0.055	0.254
NE 0.07%	2	0.24	0.007	0.030
NE 0.175%	3	0.30	0.032	0.106
NE 0.35%	2	0.46	0.000	0.000
NE 0.7%	1	0.85	NA	NA
NE 1.75%	2	1.77	0.071	0.040
NE 3.5%	2	3.49	0.240	0.069

Table 7: Analytical precision (CV) of enrichment method.

% T790M	Replicates	Average	STD	CV
E 0%	13	5.23	0.496	0.095
E 0.03%	3	7.38	0.630	0.085
E 0.07%	3	7.78	0.383	0.049
E 0.175%	4	11.00	0.413	0.038
E 0.35%	2	17.17	2.17	0.126
E 0.7%	2	27.32	4.09	0.150
E 1.75%	1	40.61	NA	NA
E 3.5%	2	62.81	8.36	0.133

Summary

These results demonstrate that we are able to detect and quantify low level frequencies of EGFR T790M in cell line and FFPE DNA. Our methods are robust and accurate, with a 0.2% LOD for the non-enrichment method, and a 0.03% LOD with the enrichment method. This highly sensitive and specific detection capability may enable earlier detection of emerging therapeutic resistance in FFPE tumor specimens. This assay can also potentially be used for detection of EGFR T790M mutations in plasma as a monitoring assay.

References

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