IMPROVEMENT OF TUMOR MUTATION BURDEN MEASUREMENT BY REMOVAL OF DEAMINATED BASES IN FFPE DNA

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ABSTRACT

Tumor mutational burden (TMB) is a positive predictive factor for response to immune-checkpoint inhibitors in certain types of cancer. The Oncomine™ Tumor Mutation Load Assay, a targeted next generation sequencing (NGS) assay, measures TMB (from 1.2Mb of coding region) and detects mutations in 409 cancer genes. The TMB values obtained using targeted sequencing are highly correlated with TMB measured by whole exome sequencing. FFPE preservation methods can lead to significant cytosine deamination of the isolated DNA, resulting in decreased sequencing quality. In these samples, uracils are propagated as thymines and result in false C>T substitutions. Analysis of the Oncomine™ TML Assay using Torrent Suite and Ion Reporter ™ software uniquely estimates the degree of deamination in fixed tissues by measuring C:G>T:A variants. This deamination score is used to assess quality of DNA extracted from FFPE tumor tissue. To minimize the influence that excess deamination has on TMB results, we have incorporated a repair treatment to eliminate damaged targets and improve usable TMB values of DNA from damaged FFPE tumor tissue using Uracil-DNA glycosylase (UDG). The Oncomine™ TML Assay for TMB on the Ion Gene Studio™ S5 systems in conjunction with a deamination score is informative and potentially predictive for the use of checkpoint inhibitors in multiple cancer types.

INTRODUCTION

Multiple FFPE repair kits are commercially available to treat genomic DNA extracted from FFPE tissues. Treatment typically involves enzymatic incubation and purification outside of the intended workflow, which can be time consuming and may result in loss of precious material. The proposed repair with UDG is integrated into the Oncomine™ TML Assay workflow, consisting of a short incubation with no purification step. This method allows for minimal intrusion of the original Oncomine™ TML Assay workflow, with no loss in sample amount.

MATERIALS AND METHODS

Cytosine deamination in FFPE DNA can propagate false C>T mutations, which can inflate tumor mutational burden results. Experiments were performed to compare TMB results of UDG treated FFPE DNA to non-treated FFPE DNA. Selection of FFPE DNA with range of TMB results and deamination scores (assigned by Ion Reporter ™ TML workflow) were chosen to test the efficacy of UDG treatment. The Ion Reporter ™ deamination score is reported as the “Estimated SNP proportion consistent with deamination (mainly FFPE)”.

Libraries from FFPE DNAs, using 20ng input total per sample, were generated with the Oncomine™ Tumor Mutation Load Assay (A37909) under the standard protocol (MAN0071042). In parallel, libraries from the same FFPE DNAs were generated under the standard protocol after UDG treatment. The UDG treatment occurs prior to the target amplification with the TML assay itself. For each FFPE DNA sample, the following components are added to a single well of a 96-well PCR plate:

Component	Volume (µL)
20ng FFPE DNA	7.3
UDG, heat-labile	1
Low TE	to 8.3

Plate is sealed and incubated in Applied Biosystems Verti ™ 96-well thermal cycler: 37ºC for 2 minutes, 50C for 10 minutes, and 4C hold for no more than 60min. UDG treated FFPE DNA is now ready to use with the standard TML Assay protocol, beginning with target amplification. Completed libraries were pooled, templated with Ion 540 ™ Kit-Chef, and sequenced on Ion Gene Studio ™ S5 Plus System with Ion 540 ™ chips.

Data analysis was completed with Torrent Suite ™ 5.10 and Ion Reporter ™ 5.10. TMB values (Mutations/Mb) was determined using Oncomine™ Tumor Mutation Load - w2.0 - DNA - Single sample workflow.

RESULTS

Table 1. Comparison of Tumor Mutational Burden Measurement (Mutations/Mb) between non-treated and UDG treated FFPE DNA

Sample	Mutations/Mb, Untreated	Deamination Score, Untreated	Mutation/Mb, UDG Treated	Deamination Score, UDG Treated
810145CN	3.38	0	3.4	0
F00092860b	5.87	14	0.84	0
F00092861b	33.84	8	31.45	0
810145CT	60.91	23	60.86	25
810136T	166.52	272	59.75	68
B810137T	305.99	618	28.18	28
B810136N	1661.09	2829	328.24	290
B810135T	5794.99	9018	800.53	610

Initial testing of UDG treatment of FFPE DNA showed reduction of mutations/Mb correspond with decrease in deamination score. UDG treatment did not affect TMB score of samples with low deamination scores and minimal damage. Initial UDG treatment experiment shows significant reduction in deamination scores, in turn reducing Mutations/Mb to a biologically relevant level. However, some samples with very high deamination scores could not be rescued as FFPE damage is too great to overcome.

Figure 1. Variant Overlap Analysis Example: FFPE Normal Sample

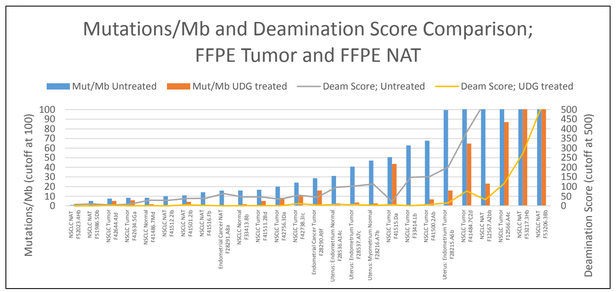
810145CN – FFPE Colon Normal Tissue. Overlap analysis of variants called, show agreement between untreated and UDG treated DNA. UDG treatment did not affect the calling of variants.

F00092860b – FFPE Colon Normal Tissue. Overlap analysis shows untreated sample with higher discordant variants, corresponding to higher deamination score. UDG treated sample generation a zero deamination score, reflective of absence of deamination.

Figure 2. Variant Overlap Analysis Example: FFPE Tumor with high deamination.

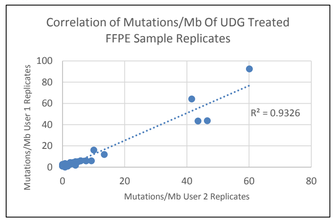
B810137T – FFPE Lung Tumor Tissue Overlap analysis shows true variants from the sample, with untreated(blue) sample showing large number of variants (627) not consistent between the two treatments. The 627 variants are likely deaminated bases that cause FPs contributing to the higher TMB. The UDG treated sample show a lower number of variants outside of the overlap, which correlates to the lower TMB.

Figure 3. Tumor Mutation Load Measurements (Mutations/Mb) Comparison: Untreated vs UDG Treatment



TMB measurement comparisons show the effectiveness of UDG across a variety of samples and range TMB measurements. Samples with TMB measurements above a score of 50 would require additional review to determine sample quality. With UDG treatment, a majority of the same samples result with Mut/Mb in a relevant range. Samples with minimal reduction show true Mut/Mb measurement with low or no deamination score.

Figure 4. FFPE DNA Repair Reproducibility



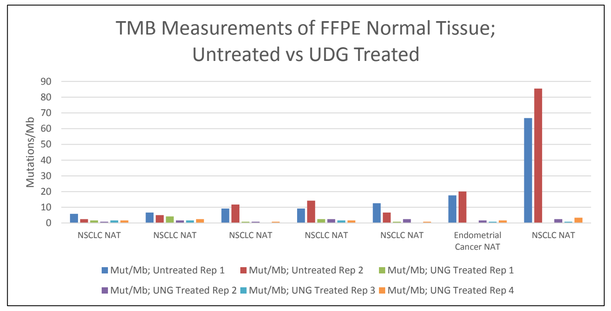
Duplicate libraries of UDG treated FFPE samples, generated across two users with different lots of library and sequencing reagents and different instrumentation, show high reproducibility (total n=4 per FFPE sample). Aggregate correlation across users show high correlation of Mut/Mb results. Study shows consistent performance of UDG treatment to repair deamination.

Table 2. Replicate Correlation

	User 1_Rep_1	User 1_Rep_2	User 2_Rep_1	User 2_Rep_2
User 1_Rep_1	1			
User 1_Rep_2	0.97	1		
User 2_Rep_1	0.98	0.91	1	
User 2_Rep_2	0.99	0.98	0.97	1

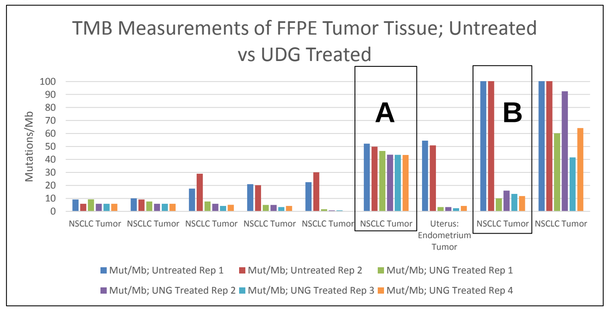
Comparison of individual replicates and users – Pearson correlation is high across users and replicates. Workflow for FFPE DNA repair show robust effect of UDG treatment.

Figure 5. UDG Treatment on FFPE Normal Tissue Repairs Deamination



FFPE Normal Adjacent to Tumor (NAT) Tissues with varying degrees of deamination, resulted in low TMB scores post UDG treatment. FPs that occur in untreated samples have been repaired and FFPE Normal Tissue TMB measurements are in-line with normal cell line TMB measurements. Replicate libraries from the same source show consistent performance of the UDG treatment to reduce deamination of FFPE damage Normal Tissues.

Figure 6. UDG Treatment on FFPE Tumor Tissue Repairs Deamination

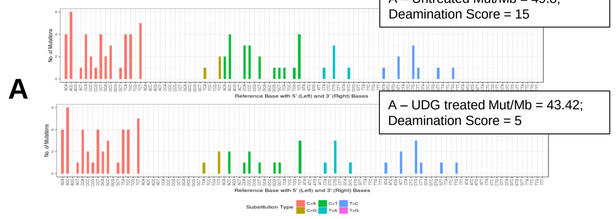


UDG treated FFPE Tumor Tissue show reduction of Mut/Mb measurement. For some samples, high TMB is only slightly reduced when associated with low deamination score to begin with (A). Tumor samples with TMB once considered too high to be relevant, has TMB reduced to a biological relevant level with UDG treatment (B).

Figure 7. Substitution Type and Context of Somatic Mutations – Sample with low deamination

A – Untreated Mut/Mb = 49.8; Deamination Score = 15

A – UDG treated Mut/Mb = 43.42; Deamination Score = 5

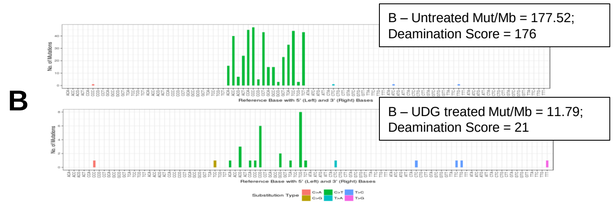


Substitution profile show minimal reduction of C>T events after UDG treatment, reflective of minimal decrease in Mut/Mb measurement. Other substitution events remained unchanged.

Figure 8. Substitution Type and Context of Somatic Mutations – Sample with high deamination

B – Untreated Mut/Mb = 177.52; Deamination Score = 176

B – UDG treated Mut/Mb = 11.79; Deamination Score = 21



Substitution profile shows effective reduction of C>T events after UDG treatment, reflective of decrease in Mut/Mb measurement. Other substitution events remained unchanged.

CONCLUSIONS

Incorporation of a repair of DNA extracted from FFPE tissues as part of the Oncomine™ TML Assay workflow show consistent and effective reduction of C>T artifacts reflective of deamination without affecting true variants. Without UDG treatment, deamination damage propagates false C>T mutations, thus inflates TMB measurements. UDG treatment corrects for deamination and provides results of TMB measurements in a biological relevant range. UDG treatment would be an effective inclusion to the Oncomine™ Tumor Mutation Load Assay workflow.

REFERENCES

1. Henn B *et al*., *Nature Reviews Genetics* 16(6) 333-343 2015 Estimating The Mutation Load in Human Genomes

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