

## RESEARCH ARTICLE

# Tumor mutational burden standardization initiatives: Recommendations for consistent tumor mutational burden assessment in clinical samples to guide immunotherapy treatment decisions

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## Funding information

F. Hoffman-La Roche AG; Merck Sharp & Dohme Ltd; Memorial Sloan Kettering Cancer Center; Johns Hopkins University; Columbia University; Thermo Fisher Scientific Inc; SeraCare Life Sciences Inc; Regeneron Pharmaceuticals Inc; QIAGEN NV; Pfizer Inc; Personal Genome Diagnostics (PGDx) Inc; OmniSeq LLC; NeoGenomics Laboratories Inc; Merck & Company Inc; Illumina Inc; Guardant Health Inc; Genentech Inc; Foundation Medicine Inc; EMD Serono Inc; Caris Life Sciences Inc; AstraZeneca LP; ACT Genomics Company Ltd; Bristol-Myers Squibb Company Inc

## Abstract

Characterization of tumors utilizing next-generation sequencing methods, including assessment of the number of somatic mutations (tumor mutational burden [TMB]), is currently at the forefront of the field of personalized medicine. Recent clinical studies have associated high TMB with improved patient response rates and survival benefit from immune checkpoint inhibitors; hence, TMB is emerging as a biomarker of response for these immunotherapy agents. However, variability in current methods for TMB estimation and reporting is evident, demonstrating a need for standardization and harmonization of TMB assessment methodology across assays and centers. Two uniquely placed organizations, Friends of Cancer Research (Friends) and the Quality Assurance Initiative Pathology (QulP), have collaborated to coordinate efforts for international multistakeholder initiatives to address this need. Friends and QulP, who have partnered with several academic centers, pharmaceutical organizations, and diagnostic companies, have adopted complementary, multidisciplinary approaches toward the goal of proposing evidence-based recommendations for achieving consistent TMB estimation and reporting in clinical samples across assays and centers. Many factors influence TMB assessment, including preanalytical factors, choice of assay, and methods of reporting. Preliminary analyses highlight the importance of targeted gene panel size and composition, and bioinformatic parameters for reliable TMB estimation. Herein, Friends and QulP propose recommendations toward consistent TMB estimation and reporting methods in clinical samples across assays and centers. These recommendations should be followed to minimize variability in TMB estimation and reporting, which will ensure reliable and reproducible identification of patients who are likely to benefit from immune checkpoint inhibitors.

## KEYWORDS

biomarkers, immune checkpoint inhibitors, neoantigens, next-generation sequencing, tumor mutational burden/load

Albrecht Stenzinger and Jeffrey D. Allen are co-first authors

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## 1 | TUMOR MUTATIONAL BURDEN AS A BIOMARKER OF RESPONSE TO IMMUNE CHECKPOINT INHIBITORS

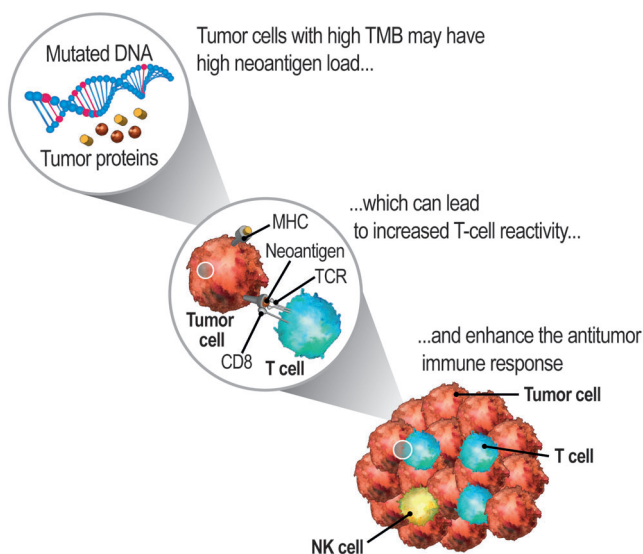
Tumor mutational burden (TMB) is the total number of somatic mutations in a defined region of a tumor genome and varies according to tumor type as well as among patients.<sup>1-4</sup> For some tumors, particularly those with high TMB, such as melanoma and lung cancers, evidence is emerging for the association of TMB with neoantigen load.<sup>2-5</sup> Neoantigens are novel tumor cell surface epitopes, some of which can be recognized as foreign to the body by the immune system, resulting in increased T-cell reactivity and thereby leading to an antitumor immune response (Figure 1).<sup>1,4,6-9</sup> Immune checkpoint inhibitors enhance antitumor T-cell activity via inhibition of immune checkpoint molecules, such as programmed death-1/programmed death ligand-1 (PD-1/PD-L1) and cytotoxic T lymphocyte antigen-4 (CTLA-4), which negatively regulate T-cell activation and contribute to tumor immune response evasion.<sup>10-12</sup> Therefore, for some tumor types, neoantigen load or TMB may be a suitable clinical biomarker to guide treatment decisions for immune checkpoint inhibitors. While not all mutations result in immunogenic neoantigens and determining which mutations are likely to induce immunogenic neoantigens remains a challenge, TMB represents a quantifiable measure of the number of mutations in a tumor that can be used to inform treatment selection.<sup>4</sup> Clinical data demonstrating that patients with tumors that have high neoantigen load or high TMB are more likely to achieve clinical benefit from treatment with immune checkpoint inhibitors are accumulating.<sup>1,13-15</sup>

Investigation of TMB as a biomarker of response to immune checkpoint inhibitors has increased over recent years. These studies have identified an association between elevated TMB and improved patient outcomes in response to anti-PD-1/PD-L1 and anti-CTLA-4 therapies in multiple tumor types.<sup>16-25</sup> Most studies to date have investigated the association of patient outcomes and TMB in patients

with non-small cell lung cancer (NSCLC). Other studies have assessed this association in patients with melanoma, squamous cell carcinoma of the head and neck, small cell lung cancer, and urothelial carcinoma. Data from retrospective or exploratory analyses indicate that TMB may be an independent biomarker for clinical efficacy of PD-1/PD-L1 and CTLA-4 inhibitors.<sup>16-20,24,26-29</sup> These observations were recently corroborated in clinical studies in patients with NSCLC treated with nivolumab in combination with ipilimumab and with atezolizumab, where high TMB (defined as  $\geq 10$  mutations per megabase [mut/Mb] and  $\geq 14$  mut/Mb, respectively) was prospectively assessed as clinically predictive for increased progression-free survival.<sup>21,23</sup> The escalation of published studies in 2017 and 2018 compared with previous years demonstrates the increased awareness of assessing TMB as a predictive marker for response to immune checkpoint inhibitors, a trend that is set to continue.

## 2 | THE FUTURE CLINICAL LANDSCAPE OF TMB

Alongside data from published studies demonstrating the association of TMB and response to immune checkpoint inhibitors, additional ongoing and planned clinical trials with a key TMB component in their design are emerging.<sup>16-25,30</sup> A search of the United States-focused ClinicalTrials.gov database (search terms "tumor mutation burden", "tumor mutational burden", "tumor mutation load", "tumor mutational load" [performed July 26, 2018]) demonstrates that the number of trials with key TMB components (defined as TMB assessment listed under study description, study design, outcome measures, or eligibility criteria) has greatly increased from 1 in 2014 to 35 in 2017, and the data for the first half of 2018 continue to follow the trend (14 trials from January 1 to July 26, 2018). Fifty-four trials were identified in the search, which have a total estimated enrollment of over 11 000 patients, and their projected primary completion dates suggest that patient TMB data will continue to accumulate through 2019 and beyond. Of the 54 trials, 37 investigate immune checkpoint inhibitors and TMB, and findings show that integration of TMB as a biomarker for response to immune checkpoint inhibitors in clinical trials is diversifying from mostly melanoma and NSCLC trials into a range of other tumor types, including endometrial, colorectal, urothelial, and breast cancers.<sup>25</sup> These findings underlie expectations that diagnostic assessment of TMB could provide benefit across many tumor types. Furthermore, as is common in the field of precision medicine, TMB assessment can be included in clinical trials as part of multiparameter assessments encompassing potential protein, DNA, and RNA biomarkers. While TMB represents one aspect of the genomic landscape, whole genome or exome and RNA sequencing may reveal functional aspects of the tumor profile, such as targetable gene mutations and/or fusions, and assessment of protein markers in the tumor microenvironment may provide additional information. Therefore, multiomic analyses may provide a more complete patient biomarker profile for guiding treatment decisions. Indeed, other biomarkers commonly investigated alongside TMB include PD-L1, microsatellite instability (MSI) or deficient mismatch repair, and immune signatures.<sup>31</sup>



**FIGURE 1** TMB association with the antitumor response. Abbreviations: CD8, cluster of differentiation 8; MHC, major histocompatibility complex; NK, natural killer; TCR, T-cell receptor

The increase in integration of TMB assessment in ongoing and upcoming clinical trials investigating immune checkpoint inhibitors demonstrates increased awareness of TMB as a potential clinical biomarker for guiding patient treatment decisions and identifying patients likely to benefit from these therapies. It also brings to the forefront the crucial need for clinicians to be aware of different TMB methodologies and reporting so that they may make informed clinical decisions.

### 3 | THE NEED FOR STANDARDIZATION AND HARMONIZATION OF TMB ASSESSMENT IN CLINICAL SAMPLES

TMB is most commonly measured by assessing formalin-fixed, paraffin-embedded (FFPE) tissue samples using next-generation sequencing (NGS) methods, whole genome sequencing (WGS), whole exome sequencing (WES), and various targeted gene panels. With advances in technology enabling targeted gene panel assays to be performed more affordably, with quick turnaround times, and with increased assay sensitivity that enables analyses of small biopsy samples or those with low tumor cellularity, such assays are increasingly being used to assess TMB, MSI status, and other genomic biomarkers.<sup>4,32,33</sup> The FDA recently granted the genomic profiling assays FoundationOne CDx and MSK-IMPACT approval and authorization, respectively, as tests for actionable mutations, copy number alterations, and fusions in solid tumors.<sup>33-36</sup> Although not yet approved for such use in the clinical setting, these assays can be used for TMB assessment. Furthermore, several targeted gene panel assays are currently being developed and validated by diagnostic companies and academic institutes, including some specific for TMB assessment in blood.

The increase in TMB assessment by various methods has brought with it a confusing array of information that documents how TMB has been determined and reported. The wide variation in TMB estimation and reporting methods across studies that have already been published demonstrates an evident lack of standardization and harmonization of current TMB assessment methods (Table 1).<sup>16-24,26-29,37-39</sup> These extensive differences may arise from the theoretic framework, technical methods applied, and the way that TMB data are reported, and will be described in more detail in the later sections of this article.

Together, the increased interest in using TMB to select patients who will most likely benefit from immune checkpoint inhibitors, increased integration of TMB assessment in ongoing clinical trials, and variability in current TMB assessment methods can create confusion for physicians and may influence critical treatment decisions. Further investigation is warranted to assess how these methods compare with one another and highlights the need for standardization and harmonization efforts for TMB estimation and reporting across assays and centers.<sup>4,40-42</sup> Standardization of TMB assessment methodology will ensure consistency of TMB estimation and reporting across assays and centers, and harmonization will enable TMB score to be more accurately compared across assays and centers. It has been recognized that the need for standardized and harmonized methodology for clinical assays can be addressed by the collaborative efforts of accredited agencies, pathologists, and oncologists. Here, we describe the critical and timely initiatives of two international organizations,

Friends of Cancer Research (Friends) and Quality Assurance Initiative Pathology (QuIP), which have collaborated to coordinate efforts to address this need for standardized and harmonized TMB estimation and reporting in clinical samples. Friends and QuIP are well placed to coordinate the international multistakeholder initiatives to understand the differences in TMB assessment methodology and propose approaches that will standardize and harmonize TMB assessment across assays and centers globally.

### 4 | FRIENDS AND QuIP TMB STANDARDIZATION AND HARMONIZATION INITIATIVES

The international collaboration between Friends and QuIP has been initiated to propose recommendations for achieving consistency in TMB estimation and reporting in clinical tissue samples across different assays, platforms, and centers (Figure 2A). Using multidisciplinary approaches, Friends and QuIP review the current methods of TMB assessment in FFPE samples and propose recommendations on how to standardize them (Figure 2B). These recommendations will inform the oncology community (including diagnostic companies, pathologists, clinicians, and the pharmaceutical industry) of best practices for TMB assessment in FFPE samples and ultimately will improve patient care by guiding treatment decisions and enabling maximum clinical benefit for patients.

Friends is a nonprofit and patient advocacy organization, founded in 1996 and based in Washington, DC, that drives collaboration between partners across diverse healthcare sectors to drive advances in science, policy, and regulation that advance treatments in patients. The organization has been instrumental in the development and implementation of policies that ensure patients quickly receive the best treatments in the safest way possible. QuIP, founded in 2004, is a joint venture between the German Society of Pathology and the German Pathologists' Association, encompassing specialists from the fields of pathology, quality management, administration, and marketing/public relations, that provides and studies pathological testing services. The organization values continued education and training for pathologists and the highest standards of quality assurance to ensure that patients receive optimal personalized treatment. Friends and QuIP are therefore uniquely positioned to perform these collaborative initiatives and provide evidence-based recommendations for reliable and reproducible TMB assessment in clinical samples across assays and centers.

Friends and QuIP have partnered with a number of academic institutes and diagnostic and pharmaceutical companies, bringing together key experts from diverse backgrounds from around the world, including pathologists, bioinformaticians, physicians, drug sponsors and regulators, diagnostic assay developers, patient advocates, and healthcare policy advisors, to achieve the coordinated goal of proposing recommendations for TMB assessment in FFPE clinical samples (Figure 2A).<sup>43,44</sup> Complementary analytical and clinical approaches have been adopted by the two organizations to provide a breadth of data to ensure that the recommendations proposed by Friends and QuIP are robust and evidence based (Figure 2B). For *in silico* analyses,

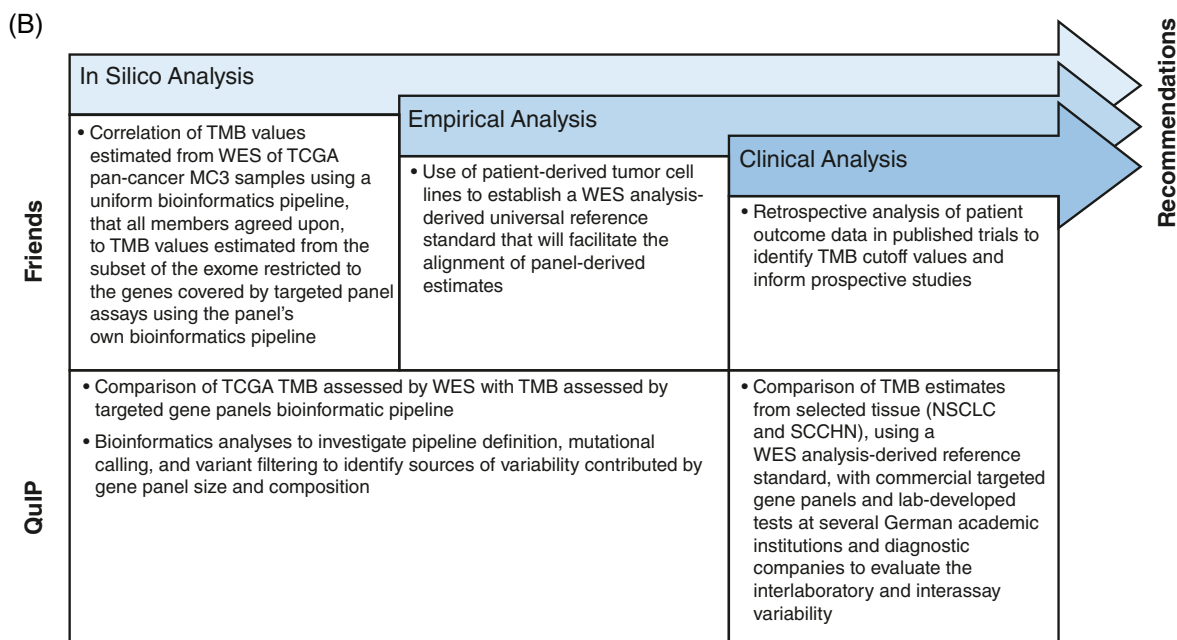
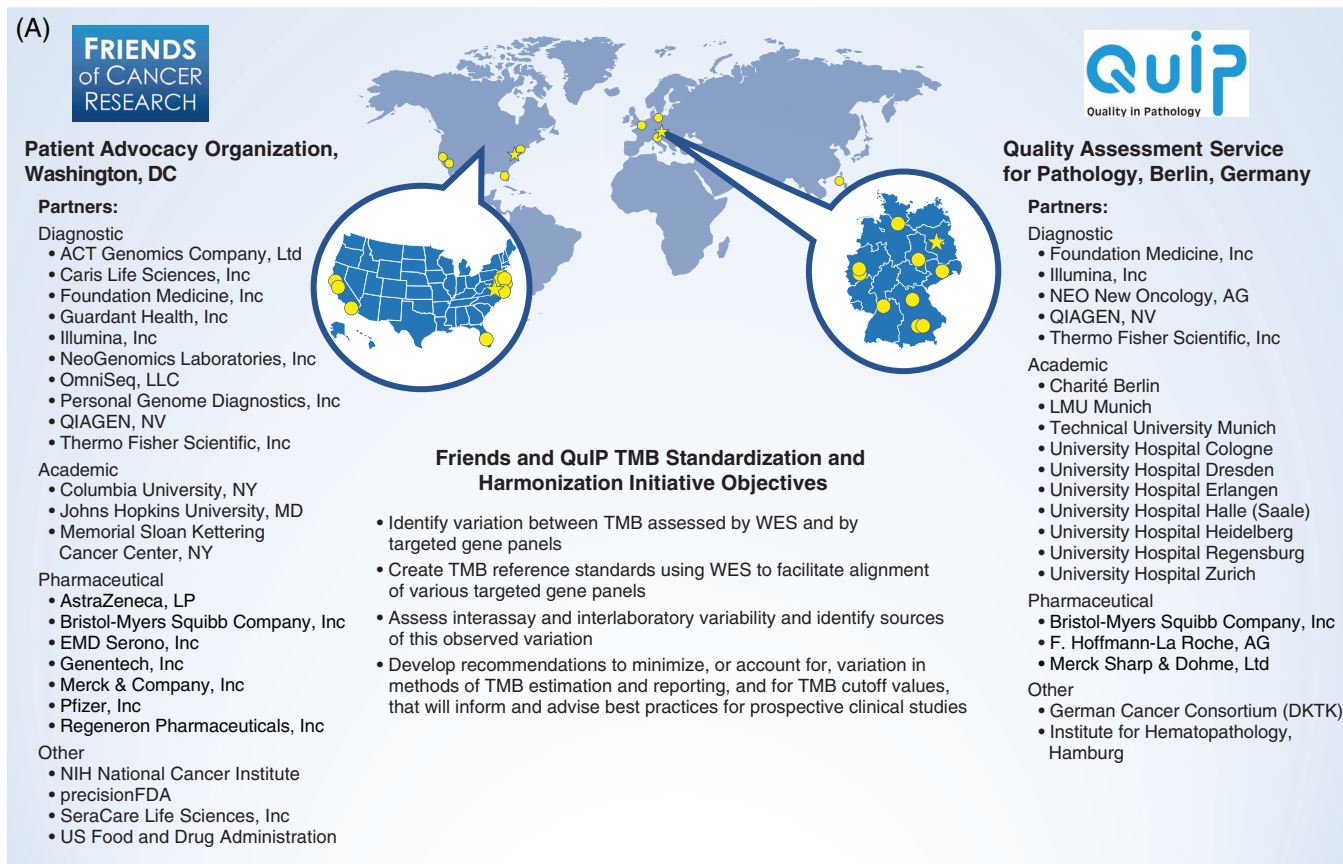
**TABLE 1** Methodology for published key trials demonstrating TMB as a biomarker of clinical response to immune checkpoint inhibitors

| Study name (NCT number)   | Tumor type and therapy agent       | Methodology  | Reporting  | Cutoff for high TMB   |
|---|------------------------------------|--|--|---|
| KEYNOTE-001 <sup>16</sup><br>(NCT01295827)  | NSCLC<br>Pembrolizumab             | WES<br><ul style="list-style-type: none"> <li>• SureSelect All Exon v2</li> <li>• Illumina HiSeq 2000</li> <li>• VAF = 10%</li> </ul>  | Somatic coding nonsynonymous mutations per exome                           | ≥178 mutations  |
| POPLAR, FIR, and BIRCH <sup>17</sup><br>(NCT02031458<br>NCT01846416<br>NCT01903993) | NSCLC<br>Atezolizumab              | FoundationOne assay <sup>4</sup><br><ul style="list-style-type: none"> <li>• 315 genes assessed</li> <li>• 1.1 Mb coverage</li> </ul>  | Somatic coding SNVs (synonymous and nonsynonymous) and indels per megabase | ≥75th percentile (≥13.5 mut/Mb for first line and ≥17.1 mut/Mb or ≥15.8 mut/Mb for second line populations) |
| CheckMate 026 <sup>18</sup><br>(NCT02041533)  | NSCLC<br>Nivolumab                 | WES<br><ul style="list-style-type: none"> <li>• AllPrep DNA isolation (tumor tissue)/QIAamp DNA isolation (blood)</li> <li>• SureSelect All Exon v5</li> <li>• Illumina HiSeq 2500</li> </ul>                        | Total somatic missense mutations per sample (tumor and blood)              | Upper tertile (≥243 mutations)  |
| KEYNOTE-012 and KEYNOTE-028 <sup>19,29</sup><br>(NCT01848834<br>NCT02054806)        | Solid tumors<br>Pembrolizumab      | WES<br>Details not specified   | Somatic coding nonsynonymous mutations per exome                           | ≥102 mutations  |
| IMvigor 210 <sup>26,27</sup><br>(NCT02108652)                                       | UC<br>Atezolizumab                 | FoundationOne assay-based panel<br><ul style="list-style-type: none"> <li>• 315 genes assessed</li> </ul>  | Somatic coding SNVs (synonymous and nonsynonymous) and indels per megabase | >16 mut/Mb  |
| POPLAR and OAK <sup>20,24</sup><br>(NCT01903993<br>NCT02008227)                     | NSCLC<br>Atezolizumab              | bTMB assay (based on the FoundationOne assay) <sup>24</sup><br><ul style="list-style-type: none"> <li>• 394 genes assessed</li> <li>• 1.1 Mb coverage</li> <li>• Illumina HiSeq 4000</li> <li>• VAF ≥0.5%</li> </ul> | Total somatic SNVs (synonymous and nonsynonymous) per assay                | ≥14 mut/Mb  |
| CheckMate 032 <sup>38</sup><br>(NCT01928394)  | SCLC<br>Nivolumab ± ipilimumab     | WES<br><ul style="list-style-type: none"> <li>• AllPrep DNA isolation (tumor tissue)/QIAamp DNA isolation (blood)</li> <li>• SureSelect All Exon v5</li> <li>• Illumina HiSeq 2500</li> </ul>                        | Somatic missense mutations per exome                                       | Upper tertile (≥248 mutations)  |
| CheckMate 012 <sup>28</sup><br>(NCT01454102)  | NSCLC<br>Nivolumab + ipilimumab    | WES<br><ul style="list-style-type: none"> <li>• SureSelect All Exon v2, v4, or Nextera Rapid Capture Exome kit</li> <li>• Illumina HiSeq 2000, 2500, or 4000</li> <li>• VAF = 5%</li> </ul>                          | Nonsynonymous mutations (SNVs or indels) per exome                         | Upper tertile (not specified), median (>158 mutations), or upper quartile (≥307 mutations)                  |
| CheckMate 038 <sup>39</sup><br>(NCT01621490)  | Melanoma<br>Nivolumab ± ipilimumab | WES<br><ul style="list-style-type: none"> <li>• SureSelect All Exon v2</li> <li>• Illumina HiSeq 2000 or 2500</li> <li>• Allele read count ≥5</li> </ul>   | Nonsynonymous mutations (SNVs or indels) per exome                         | 100 mutations   |
| CheckMate 275 <sup>37</sup><br>(NCT02387996)  | UC<br>Nivolumab                    | WES<br><ul style="list-style-type: none"> <li>• Details not specified</li> </ul>   | Somatic missense mutations per tumor                                       | Upper tertile (≥167 mutations)  |
| CheckMate 227 and CheckMate 568 <sup>21,22</sup><br>(NCT02477826<br>NCT02659059)    | NSCLC<br>Nivolumab and ipilimumab  | FoundationOne CDx assay <sup>34</sup><br><ul style="list-style-type: none"> <li>• 324 genes assessed</li> <li>• 0.8 Mb coverage</li> <li>• Illumina HiSeq 4000</li> <li>• VAF = 5%</li> </ul>                        | Somatic SNVs (synonymous and nonsynonymous) and indels per megabase        | ≥10 mut/Mb  |
| B-F1RST <sup>23,24</sup><br>(NCT02848651)   | NSCLC<br>Atezolizumab              | bTMB assay (based on FoundationOne)<br><ul style="list-style-type: none"> <li>• 394 genes assessed</li> <li>• 1.1 Mb coverage</li> <li>• Illumina HiSeq 4000</li> <li>• VAF ≥0.5%</li> </ul>                         | Total somatic SNVs (synonymous and nonsynonymous) per assay                | ≥14 mut/Mb  |

Abbreviations: bTMB, blood tumor mutational burden; indels, short insertions and deletions; mut/Mb, mutations per megabase; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; SNV, single nucleotide variant; TMB, tumor mutational burden; UC, urothelial carcinoma; VAF, variant allele frequency; WES, whole exome sequencing

both Friends and QuIP have utilized publicly available data from The Cancer Genome Atlas to compare TMB values derived using WES, which is currently considered as the gold standard for calculating

TMB, with those calculated using targeted gene panels. Both these approaches have focused on identifying factors that contribute to variation in TMB calculation and harmonizing bioinformatic pipelines.

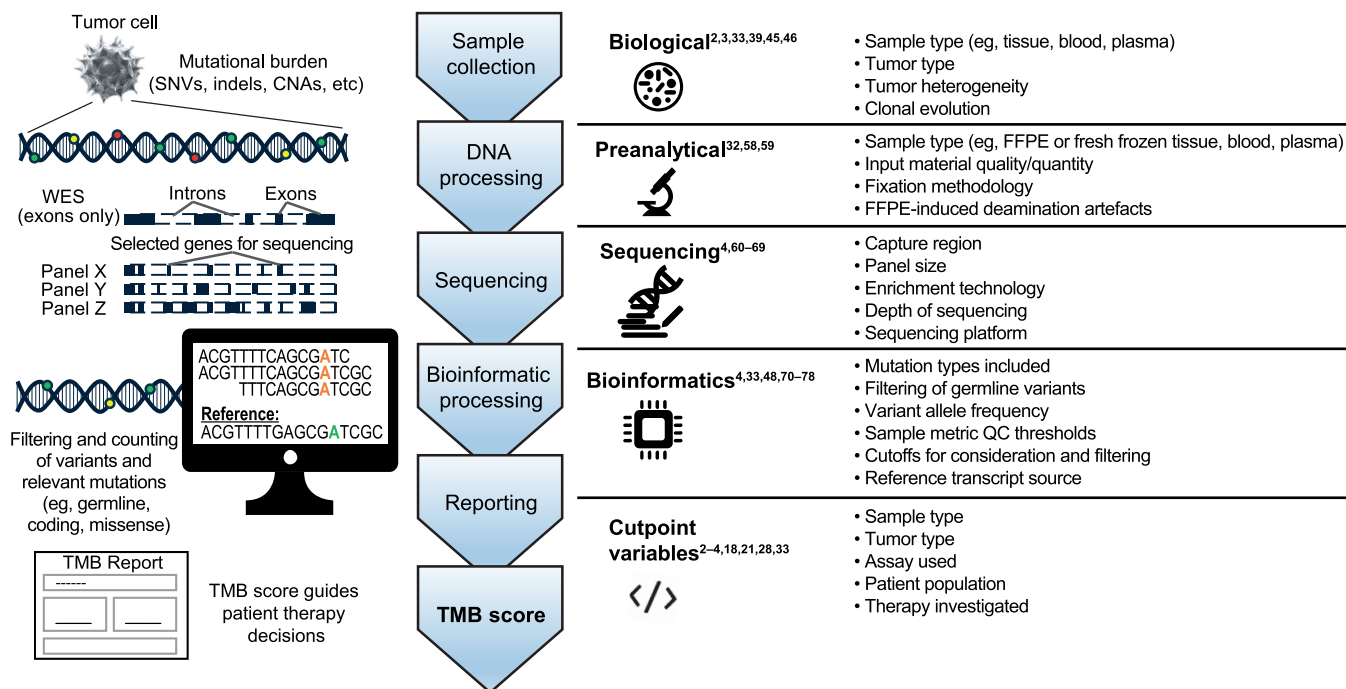


**FIGURE 2** A, Objectives and partners, and B, methodological approaches adopted for the collaborative Friends and QuIP TMB standardization and harmonization initiatives. Abbreviations: Friends, Friends of Cancer Research; FDA, Food and Drug Administration; MC3, Multi-Center Mutation Calling in Multiple Cancers; NSCLC, non-small cell lung cancer; QuIP, Quality Assurance Initiative Pathology; SCCHN, squamous cell carcinoma of the head and neck; TCGA, The Cancer Genome Atlas; TMB, tumor mutational burden; WES, whole exome sequencing

Both organizations will then develop TMB reference standards, with Friends using commercially available tumor cell lines and QuIP using clinical samples, that will facilitate the alignment of TMB assessed by WES and by targeted gene panels. As part of clinical analyses, QuIP will compare TMB assessment across assays and centers to provide

recommendations to minimize interassay and interlaboratory variation in TMB estimation and reporting. The targeted panel assays being tested by QuIP include ThermoFisher OncoPrint Tumor Mutation Load Assay, QIAGEN QIAseq Targeted DNA IO Panel, QIAGEN QIAseq Targeted DNA Booster Panel, NEO NewOncology NEOplus RUO,





**FIGURE 3** Factors that impact TMB or TMB estimation and reporting throughout the TMB assessment process. Abbreviations: CNA, copy number alteration; FFPE, formalin-fixed, paraffin-embedded; indels, short insertions and deletions; QC, quality control; SNV, single nucleotide variant; TMB, tumor mutational burden; WES, whole exome sequencing

Foundation Medicine FoundationOne Panel, Illumina TruSight Oncology 500, and several laboratory-derived assay panels developed in German academic institutes. The Friends initiative evaluates 11 TMB platforms and assays with different TMB assessment parameters, including the FoundationOne CDx and MSK-IMPACT assays, to provide an overview of how these panels compare with one another and to highlight how different factors can influence TMB estimation and reporting. As part of clinical analyses, Friends will evaluate TMB cutoff values in published studies to propose recommendations to inform prospective clinical studies.

Together, data from these multidisciplinary TMB standardization and harmonization approaches cover a wide spectrum of critical aspects of TMB assessment to propose recommendations for consistent TMB estimation, assay comparability, and TMB cutoff values for potential clinical use.<sup>43</sup>

## 5 | VARIATION IN TMB ASSESSMENT AND FACTORS THAT IMPACT TMB OUTPUT




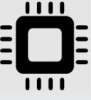

Review of the published literature indicates that several factors influence TMB assessment, and results of preliminary analyses from the Friends and QuIP initiatives indicate that certain factors have greater impact than others on TMB estimation and reporting; as summarized in Figure 3 and Table 2, and discussed below.

Biological parameters result in differences between overall tumor mutational frequency, with the most basic being tumor type and sample type.<sup>2,3,32</sup> Alexandrov et al. observed that TMB varies according to tumor type, with some tumors intrinsically having higher TMB than others.<sup>2</sup> For example, melanoma and lung tumors have higher TMB than renal carcinomas, brain-related tumors, and hematological

cancers. TMB can also be affected by tumor cellularity and heterogeneity, with subclonal events having a higher impact on mutational burden than clonal evolution.<sup>3,39,45,46</sup> Additionally, tumor transcriptional and/or splicing profiles may differ from reference profiles, resulting in miscounts and impacting the TMB score reported.<sup>47,48</sup>




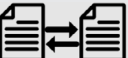
To date, most of the published studies have assessed TMB in solid tumor samples; however, blood TMB assessment assays are increasingly being used to assess TMB association with response to immune checkpoint inhibitors (Table 1). Because TMB is most commonly assessed using FFPE tumor tissue samples, the initiative by Friends and QuIP proposes recommendations for standardized TMB assessment in these samples; however, TMB assessment using liquid samples is being evaluated by many other groups. Currently, there are several limitations to using other samples for TMB assessment, including that due to low levels of circulating tumor DNA (ctDNA), liquid samples may not yield sufficient quantity for NGS analysis.<sup>24,49–53</sup> Reports show that the sensitivity and accuracy of TMB assay results from liquid samples depend on, among other factors, variability in tumor DNA in the blood. ctDNA can have heterogeneous origins and can be altered by treatment, thereby leading to variation in the final TMB score.<sup>24,49–53</sup> Several ongoing studies are evaluating reliability of TMB assessment from blood samples and harmonizing tissue and blood-derived TMB, including use of the bTMB assay developed by Foundation Medicine.<sup>20,24,53,54</sup> The potential limitations of specificity, sensitivity, and robustness of TMB assessment using blood samples should be further investigated and appropriate guidance should be given on how to address such limitations. Similarly, genome profiling in cytology samples requires a minimum level of cellularity and tumor content, and use for TMB assessment should be further investigated.<sup>55</sup>

**TABLE 2** How factors impact TMB score

| Factor   | Select parameter/<br>technical consideration | Impact on TMB score   |
|--|--|---|
| Biological<br>            | Tumor type                                   | Alternative splicing patterns are dependent on tumor types, and some tumor types have higher TMB than others <sup>2,47</sup>  |
| Preanalytical<br>         | Sample type                                  | FFPE samples may harbor artefactual deamination alterations that may impact mutation calling and TMB calculation <sup>56,57</sup>   |
|  | Tumor purity                                 | Infiltration of tumor with immune or TME cells may impact TMB score (lower tumor purity is associated with reduced sensitivity) <sup>32</sup>   |
| Sequencing parameters<br> | Genomic region covered                       | TMB score will depend on panel size and genomic region covered. Greater panel sizes are associated with more precise TMB estimated values <sup>4,62-69</sup>  |
|  | Genes included in panel                      | Gene selection in panels is biased toward frequently mutated cancer-associated genes, and mutation patterns of these genes are often nonrandom. <sup>33</sup> TMB scores may depend on whether the panel contains specific genes that harbor frequent mutations in specific tumor types |
|  | Depth of coverage                            | Reduced depth of coverage is associated with reduced sensitivity <sup>33,61</sup>   |
| Bioinformatics<br>        | Germline variant removal/filtration          | Major germline genomic databases have different population race distribution and allele frequency spectrum of variants. TMB score will depend on selection of population allele frequency database when matched tumor-normal tissue is not available <sup>4</sup>                       |
|  | Reference transcript source                  | The choice of reference transcript source may impact TMB score depending on the variants considered and counted <sup>48</sup>   |
|  | Variants counted in TMB calculation          | Panels may consider all variant types or only some of them during their TMB calculations. <sup>1,4,33</sup> TMB score will depend on how comprehensive the variant counting rules are   |
|  | Mutation callers                             | Mutation callers will count variants differently, with some being more comprehensive than others. <sup>71</sup> There is no optimal mutation caller, so a combination of different callers may be most optimal  |
|  | Allele frequency/fraction                    | Reduced variant allele fraction is associated with reduced sensitivity <sup>74</sup>  |
|  | Minimum variant count                        | Reduced variant counts are associated with reduced sensitivity <sup>62</sup>  |
| Cutoff variables<br>    | Tumor type                                   | TMB differs widely across tumor types. The cutoff chosen must be appropriate for the tumor type being tested for a reliable and clinically meaningful TMB score to define high TMB <sup>2-5</sup>   |

Abbreviations: FFPE, formalin-fixed, paraffin-embedded; TMB, tumor mutational burden; TME, tumor microenvironment

**TABLE 3** Proposed recommendations for consistent TMB assessment

| Factor   | Parameter                   | Recommendations   |
|--|-----------------------------|---|
| Preanalytical<br>         | Sample processing           | <ul style="list-style-type: none"> <li>Standardize sample processing protocols</li> <li>Minimize interlaboratory variability</li> </ul>   |
| Sequencing parameters<br> | Genomic region covered      | <ul style="list-style-type: none"> <li>Select gene panels that screen for actionable mutations or biomarkers</li> <li>Select panels with larger genome coverage (ideally ~1 megabase or greater)</li> </ul>   |
| Bioinformatics<br>        | Standardization of workflow | <ul style="list-style-type: none"> <li>Align panel-derived TMB values to a WES analysis-derived reference standard to ensure consistency regardless of the assay</li> <li>Standardize bioinformatic algorithms used for mutation calling and filtering</li> </ul> |
| Comparison of results<br> | Calibration of outputs      | <ul style="list-style-type: none"> <li>Ensure reporting consistency by developing templates for clinically meaningful reporting (eg, report TMB as mutations per megabase)</li> <li>Allow calibration of results from different studies</li> </ul>                |

Abbreviations: TMB, tumor mutational burden; WES, whole exome sequencing

TMB estimation and reporting can be heavily influenced by differing working processes across clinical and research laboratories; primarily, the choice of assay, platform, and how the assay is implemented.<sup>32</sup> Preanalytical factors can also have significant effects on TMB estimation, including those that apply to all genomic profiling assays, such as sample collection and processing, input material quality and quantity, sample fixation methodology, FFPE-induced deamination artefacts, and NGS library preparation.<sup>56,57</sup> These factors affect the quantity and quality of DNA extracted for TMB assessment by either WES or targeted gene panel assays, and therefore, TMB estimation output. For example, low tumor purity, which can result from infiltration of immune or tumor microenvironment cells, can lead to reduced TMB assay sensitivity. Also, fixation time is a preanalytical factor that influences the introduction of FFPE-induced deamination artefacts, which also impacts TMB estimation at the stage of bioinformatic analysis.<sup>58,59</sup>

For sequencing, genome coverage differs between WGS, WES, and targeted gene panel assays. WGS covers the whole genome, WES covers the entire exome coding region, and targeted gene panels cover specified areas that may or may not include tumor suppressor genes, driver genes, or intronic regions.<sup>4,60,61</sup> Moreover, the size and location of the capture region differs between targeted gene panel assays. It is important to carefully consider the panel size and composition for accurate TMB assessment. Supporting this concept, it has been observed that confidence intervals for TMB estimation increase with the use of gene panels that assess a smaller area of the genome compared with those that assess a larger area, which suggests that using smaller coverage gene panels could lead to the overestimation or underestimation of TMB.<sup>4,62-69</sup> Depth of sequencing also differs between WES and targeted gene panel assays; sequencing depth is greater for targeted gene panels (~500x) than for WES (~100x).<sup>4,61,64</sup> Genome coverage and sequencing depth together determine assay sensitivity and specificity, and therefore, influence TMB estimation output.

Bioinformatic algorithms can differ widely across targeted gene panels and although these factors heavily influence TMB estimation and reporting, the specifics are often not reported (Table 1). The mutation types considered for TMB assessment can vary from one assay to another. These may include or exclude short insertions and deletions (indels) and/or synonymous and nonsynonymous base substitutions/single nucleotide variants.<sup>4,33,70</sup> For example, from retrospective analyses, it has been observed that TMB assessed by WES often includes missense mutations only, leaving out indels and other mutations, whereas some targeted panels include these variant types.<sup>4,18,20,21,24,26,27</sup> This is an important consideration due to the impact of indels and frameshift mutations on neoantigen formation.<sup>4</sup> However, calling indels can be challenging and their inclusion may depend on the sensitivity of the methods used to detect them.<sup>71</sup> Other bioinformatic parameters that impact TMB estimation and reporting include quality control metrics and various data-filtering procedures for inclusion/exclusion of a variant in the TMB estimation.<sup>4,18,33,38,48</sup> Filtering algorithms and cutoffs for putative germline variants, variant allele frequency (VAF), and FFPE-induced deamination artefacts vary between assays and can be affected by biological and preanalytical factors. For example, VAF cutoffs can vary from 0.5 to 10%, with lower thresholds increasing the risk of including false-positives arising from contamination or sequencing artefacts.<sup>4,24,36,48,72-74</sup>

For calculation of the TMB denominator, genome coverage and bioinformatic parameters must be considered. Most studies have reported a TMB value in mut/Mb, whereas others have reported total mutations per tumor (WES studies); this makes it difficult to compare TMB values across patients and studies. Alongside the way in which TMB is reported, a key factor that must be aligned to ensure consistent identification of patients who are likely to benefit from immune checkpoint inhibitors, and which is currently variable among assays and centers, is the cutoff threshold that defines tumor TMB as high or low. Cutoffs may differ depending on sample type, tumor type, patient subgroup, therapy investigated, and assay used, and the recommendations proposed by Friends and QuIP aim to facilitate the identification of such cutoffs to inform prospective clinical studies.<sup>2-4,18,21,28,32</sup>

## 6 | RECOMMENDATIONS FOR RELIABLE TMB ESTIMATION AND REPORTING IN CLINICAL SAMPLES

The Friends and QuIP initiatives have proposed recommendations for the standardization of TMB assessment to improve reproducibility and reliability, and best practices for how to minimize and account for variability among assays (Table 3). From results of preliminary analyses, we recommend that NGS assays provide as much patient-relevant genetic/molecular information as possible to avoid the need for rebiopsy and retesting of quality samples at baseline. This will be critical to guide immediate therapy selection with targeted therapies. For example, testing of actionable driver mutations (eg, EGFR inhibitor therapies for EGFR-mutated lung cancers), genes associated with mutagenesis (eg, POLE), and potential negative predictors of response (eg, mutated  $\beta$ 2M, JAK1/2, PTEN, STK11).<sup>75-78</sup> We recommend that targeted gene panel assays that have larger genome coverage (ideally with ~1 megabase being the lower limit) are used because they yield more reliable TMB estimation than smaller panels.<sup>67-69</sup> Of note, panels that cover less than 1 megabase are useful; however, accuracy may be reduced.<sup>67-69</sup> We also recommend the use of external reference sequence data, generated using agreed standard methodology such as WES, as this may enable and facilitate TMB assessment interpretability across panel assays.

Ongoing empirical and clinical analyses to generate reference standards, compare TMB measured by WES with TMB measured by various targeted gene panels, and evaluate and minimize interlaboratory and interassay variability are underway. These data will investigate additional aspects of TMB measurement to ensure consistency between assays and laboratories, based on the expectation that many laboratories may develop their own tests for TMB assessment.

## 7 | CONCLUSIONS

Standardization and harmonization of TMB assessment across assays and centers are essential for reliable and reproducible use of TMB as a clinical biomarker of response to immune checkpoint inhibitors. There is a recent increase in the integration of TMB as a biomarker to select patients who will most likely benefit from immune checkpoint



inhibitors in clinical trials. Increased use of TMB, as well as the current variations in methods of TMB estimation and reporting, highlights the need for standardized and harmonized methods for TMB assessment.

Results of preliminary analyses from Friends and QuIP highlight the importance of targeted gene panel size and composition, and bioinformatic pipeline for reliable TMB estimation in FFPE samples. Following the critical and timely recommendations proposed by Friends and QuIP will help minimize variability in TMB estimation and reporting, which will ensure consistency of TMB assessment in clinical samples across assays and centers. This will improve interpretability of TMB data across assays and studies and lead to the more reliable and accurate use of TMB as a biomarker to identify patients likely to benefit from immune checkpoint inhibitors and to effectively guide patient treatment decisions.

## ACKNOWLEDGMENTS

Medical writing support and editorial assistance were provided by Amrita Dervan, MSc, and Jay Rathi, MA, of Spark Medica Inc, according to Good Publication Practice guidelines, funded by Bristol-Myers Squibb. The Friends and QuIP TMB assessment standardization and harmonization initiative studies are supported by several participating organizations and companies, including Bristol-Myers Squibb Company Inc (Friends and QuIP), ACT Genomics Company Ltd, AstraZeneca LP, Caris Life Sciences Inc, EMD Serono Inc, Foundation Medicine Inc, Genentech Inc, Guardant Health Inc, Illumina Inc, Merck & Company Inc, NeoGenomics Laboratories Inc, OmniSeq LLC, Personal Genome Diagnostics (PGDx) Inc, Pfizer Inc, QIAGEN NV, Regeneron Pharmaceuticals Inc, SeraCare Life Sciences Inc, Thermo Fisher Scientific Inc, Columbia University, Johns Hopkins University, Memorial Sloan Kettering Cancer Center (Friends), Merck Sharp & Dohme Ltd, and F. Hoffman-La Roche AG (QuIP). The Friends initiative utilizes a distributed research model and costs incurred are borne by each participating organization.

## CONFLICT OF INTEREST

MD reports honoraria from Bristol-Myers Squibb. JM reports honoraria for talks from Bristol-Myers Squibb. AS reports advisory board fees from AstraZeneca, Bayer, Bristol-Myers Squibb, Illumina, Novartis, and Thermo Fisher Scientific, and honoraria for talks from AstraZeneca, Bristol-Myers Squibb, MSD, Illumina, Novartis, Roche, Bayer, and Thermo Fisher Scientific. JDA, DMM, MDS, and MMW declare no conflicts of interest.

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## REFERENCES

- Schumacher TN, Schreiber RD. Neoantigens in cancer immunotherapy. *Science*. 2015;348(6230):69-74.
- Alexandrov LB, Nik-Zainal S, Wedge DC, et al. Signatures of mutational processes in human cancer. *Nature*. 2013;500(7463):415-421.
- Lawrence MS, Stojanov P, Polak P, et al. Mutational heterogeneity in cancer and the search for new cancer-associated genes. *Nature*. 2013;499(7457):214-218.
- Chalmers ZR, Connelly CF, Fabrizio D, et al. Analysis of 100,000 human cancer genomes reveals the landscape of tumor mutational burden. *Genome Med*. 2017;9(1):34.
- Chen YP, Zhang Y, Lv JW, et al. Genomic analysis of tumor microenvironment immune types across 14 solid cancer types: immunotherapeutic implications. *Theranostics*. 2017;7(14):3585-3594.
- Kim JM, Chen DS. Immune escape to PD-L1/PD-1 blockade: seven steps to success (or failure). *Ann Oncol*. 2016;27(8):1492-1504.
- Stratton MR, Campbell PJ, Futreal PA. The cancer genome. *Nature*. 2009;458:719-724.
- Chabanon RM, Pedrero M, Lefebvre C, Marabelle A, Soria JC, Postel-Vinay S. Mutational landscape and sensitivity to immune checkpoint blockers. *Clin Cancer Res*. 2016;22(17):4309-4321.
- Giannakis M, Mu Ximeng J, Shukla Sachet A, et al. Genomic correlates of immune-cell infiltrates in colorectal carcinoma. *Cell Rep*. 2016;15(4):857-865.
- Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer*. 2012;12(4):252-264.
- Iwai Y, Ishida M, Tanaka Y, Okazaki T, Honjo T, Minato N. Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proc Natl Acad Sci U S A*. 2002;99(19):12293-12297.
- Nobel Media AB. The Nobel Prize in Physiology or Medicine 2018. <https://www.nobelprize.org/prizes/medicine/2018/press-release/>. Accessed October 2, 2018.
- Yarchoan M, Hopkins A, Jaffee EM. Tumor mutational burden and response rate to PD-1 inhibition. *N Engl J Med*. 2017;377(25):2500-2501.
- Liontos M, Anastasiou I, Bamias A, Dimopoulos M-A. DNA damage, tumor mutational load and their impact on immune responses against cancer. *Ann Transl Med*. 2016;4(14):264.
- Sharma P, Allison JP. The future of immune checkpoint therapy. *Science*. 2015;348(6230):56-61.
- Rizvi NA, Hellmann MD, Snyder A, et al. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. *Science*. 2015;348(6230):124-128.
- Kowanetz M, Zou W, Shames D, et al. Tumor mutation burden (TMB) is associated with improved efficacy of atezolizumab in 1L and 2L+ NSCLC patients. *J Thorac Oncol*. 2017;12(1):S321-S322.
- Carbone DP, Reck M, Paz-Ares L, et al. First-line nivolumab in stage IV or recurrent non-small-cell lung cancer. *N Engl J Med*. 2017;376(25):2415-2426.
- Cristescu R, Mogg R, Ayers M, et al. Mutational load (ML) and T-cell-inflamed microenvironment as predictors of response to pembrolizumab. *J Clin Oncol*. 2017;35(suppl 7S): Abstract 1. Available at [http://ascopubs.org/action/showCitFormats?doi=10.1200/JCO.2017.35.7\\_suppl.1](http://ascopubs.org/action/showCitFormats?doi=10.1200/JCO.2017.35.7_suppl.1).
- Fabrizio D, Lieber D, Malboeuf C, et al. A blood-based next-generation sequencing assay to determine tumor mutational burden (bTMB) is associated with benefit to an anti-PD-L1 inhibitor, atezolizumab. Presented at: AACR Annual Meeting; April 14-18, 2018; Chicago, IL. *Cancer Res*. 2018;78(13 suppl). Abstract 5706.
- Hellmann MD, Ciuleanu TE, Pluzanski A, et al. Nivolumab plus ipilimumab in lung cancer with a high tumor mutational burden. *N Engl J Med*. 2018;378(22):2093-2104.
- Ramalingam SS, Hellmann MD, Awad MM, et al. Tumor mutational burden (TMB) as a biomarker for clinical benefit from dual immune checkpoint blockade with nivolumab (nivo) + ipilimumab (ipi) in first-line (1L) non-small cell lung cancer (NSCLC): identification of TMB cutoff from CheckMate 568. *Cancer Res*. 2018;78(suppl 13): CT078. Available at [http://cancerres.aacrjournals.org/content/78/13\\_Supplement/CT078](http://cancerres.aacrjournals.org/content/78/13_Supplement/CT078).
- Velcheti V, Kim ES, Mekhail T, et al. Prospective clinical evaluation of blood-based tumor mutational burden (bTMB) as a predictive biomarker for atezolizumab (atezo) in 1L non-small cell lung cancer (NSCLC): interim B-F1RST results. *J Clin Oncol*. 2018;36(suppl): 12001. Available at [http://ascopubs.org/action/showCitFormats?doi=10.1200/JCO.2018.36.15\\_suppl.12001](http://ascopubs.org/action/showCitFormats?doi=10.1200/JCO.2018.36.15_suppl.12001).
- Gandara DR, Paul SM, Kowanetz M, et al. Blood-based tumor mutational burden as a predictor of clinical benefit in non-small-cell lung cancer patients treated with atezolizumab. *Nat Med*. 2018;24(9):1441-1448.

25. Chan TA, Yarchoan M, Jaffee E, et al. Development of tumor mutation burden as an immunotherapy biomarker: utility for the oncology clinic. *Ann Oncol*. 2018;30(1):44-56.
26. Rosenberg JE, Hoffman-Censits J, Powles T, et al. Atezolizumab in patients with locally advanced and metastatic urothelial carcinoma who have progressed following treatment with platinum-based chemotherapy: a single-arm, multicentre, phase 2 trial. *Lancet*. 2016;387(10031):1909-1920.
27. Balar AV, Galsky MD, Rosenberg JE, et al. Atezolizumab as first-line treatment in cisplatin-ineligible patients with locally advanced and metastatic urothelial carcinoma: a single-arm, multicentre, phase 2 trial. *Lancet*. 2017;389(10064):67-76.
28. Hellmann MD, Nathanson T, Rizvi H, et al. Genomic features of response to combination immunotherapy in patients with advanced non-small-cell lung cancer. *Cancer Cell*. 2018;33(5):843-852.
29. Helwick C. KEYNOTE trial data suggest features predicting response to pembrolizumab; 2017. <http://www.ascopost.com/issues/march-25-2017/keynote-trial-data-suggest-features-predicting-response-to-pembrolizumab/>. Accessed October 10, 2018.
30. NIH. ClinicalTrials.gov. <https://clinicaltrials.gov/ct2/home>. Accessed October 10, 2018.
31. Cristescu R, Mogg R, Ayers M, et al. Pan-tumor genomic biomarkers for PD-1 checkpoint blockade-based immunotherapy. *Science*. 2018;362(6411):eaar3593.
32. Chen H, Luthra R, Goswami RS, Singh RR, Roy-Chowdhuri S. Analysis of pre-analytic factors affecting the success of clinical next-generation sequencing of solid organ malignancies. *Cancers (Basel)*. 2015;7(3):1699-1715.
33. Zehir A, Benayed R, Shah RH, et al. Mutational landscape of metastatic cancer revealed from prospective clinical sequencing of 10,000 patients. *Nat Med*. 2017;23(6):703-713.
34. FDA. FoundationOne CDx: summary of safety and effectiveness data (SSED); 2017. [https://www.accessdata.fda.gov/cdrh\\_docs/pdf17/P170019B.pdf](https://www.accessdata.fda.gov/cdrh_docs/pdf17/P170019B.pdf). Accessed October 10, 2018.
35. FDA. FDA announces approval, CMS proposes coverage of first breakthrough-designated test to detect extensive number of cancer biomarkers; 2017. <https://www.fda.gov/newsevents/newsroom/pressannouncements/ucm587273.htm>. Accessed October 10, 2018.
36. FDA. FDA unveils a streamlined path for the authorization of tumor profiling tests alongside its latest product action; 2017. <https://www.fda.gov/newsevents/newsroom/pressannouncements/ucm585347.htm>. Accessed October 10, 2018.
37. Galsky MD, Saci A, Szabo PM, et al. Impact of tumor mutation burden on nivolumab efficacy in second-line urothelial carcinoma patients: exploratory analysis of the phase II CheckMate 275 study. *Ann Oncol*. 2017;28(suppl 5): Abstract 848PD. Available at <https://oncologypro.esmo.org/Meeting-Resources/ESMO-2017-Congress/Impact-of-Tumor-Mutation-Burden-on-Nivolumab-Efficacy-in-Second-Line-Urothelial-Carcinoma-Patients-Exploratory-Analysis-of-the-Phase-II-CheckMate-275-Study>.
38. Hellmann MD, Callahan MK, Awad MM, et al. Tumor mutational burden and efficacy of nivolumab monotherapy and in combination with ipilimumab in small-cell lung cancer. *Cancer Cell*. 2018;33(5):853-861.
39. Riaz N, Havel JJ, Makarov V, et al. Tumor and microenvironment evolution during immunotherapy with nivolumab. *Cell*. 2017;171(4):934-949.
40. Deans ZC, Costa JL, Cree I, et al. Integration of next-generation sequencing in clinical diagnostic molecular pathology laboratories for analysis of solid tumours; an expert opinion on behalf of IQN Path ASBL. *Virchows Arch*. 2017;470(1):5-20.
41. IQN Path. International quality network for pathology. Annual report; 2017. [http://www.iqnpath.org/wp-content/uploads/2018/04/IQNPath\\_AnnualReport2017-26032018.pdf](http://www.iqnpath.org/wp-content/uploads/2018/04/IQNPath_AnnualReport2017-26032018.pdf). Accessed October 10, 2018.
42. van Krieken H, Deans S, Hall JA, Normanno N, Ciardiello F, Douillard JY. Quality to rely on: meeting report of the 5th Meeting of External Quality Assessment, Naples 2016. *ESMO Open*. 2016;1(5):e000114.
43. Friends of Cancer Research. Tumor mutational burden (TMB); 2018. <https://www.focr.org/tmb>. Accessed October 10, 2018.
44. Enzlinger N. Tumor mutational burden (TMB): QulP organises a study and liaises with FoCR; 2018. [https://wp.quip.eu/en\\_GB/2018/05/14/tumor-mutational-burden-tmb-quip-organisiert-studie-und-arbeitet-mit-focr-zusammen/](https://wp.quip.eu/en_GB/2018/05/14/tumor-mutational-burden-tmb-quip-organisiert-studie-und-arbeitet-mit-focr-zusammen/). Accessed October 10, 2018.
45. McGranahan N, Furness AJ, Rosenthal R, et al. Clonal neoantigens elicit T cell immunoreactivity and sensitivity to immune checkpoint blockade. *Science*. 2016;351(6280):1463-1469.
46. Gerlinger M, Rowan AJ, Horswell S, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N Engl J Med*. 2012;366(10):883-892.
47. Kahles A, Lehmann KV, Toussaint NC, et al. Comprehensive analysis of alternative splicing across tumors from 8,705 patients. *Cancer Cell*. 2018;34(2):211-224.
48. Duzkale H, Shen J, McLaughlin H, et al. A systematic approach to assessing the clinical significance of genetic variants. *Clin Genet*. 2013;84(5):453-463.
49. Davis AA, Chae YK, Agte S, et al. Comparison of tumor mutational burden (TMB) across tumor tissue and circulating tumor DNA (ctDNA). *J Clin Oncol*. 2017;35(suppl 15):e23028-e23028.
50. Ma M, Zhu H, Zhang C, et al. "Liquid biopsy"—ctDNA detection with great potential and challenges. *Ann Transl Med*. 2015;3(16):235.
51. Rolfo C, Mack PC, Scagliotti GV, et al. Liquid biopsy for advanced non-small cell lung cancer (NSCLC): a statement paper from the IASLC. *J Thorac Oncol*. 2018;13(9):1248-1268.
52. Yang N, Li Y, Liu Z, et al. The characteristics of ctDNA reveal the high complexity in matching the corresponding tumor tissues. *BMC Cancer*. 2018;18:319.
53. Merker JD, Oxnard GR, Compton C, et al. Circulating tumor DNA analysis in patients with cancer: American Society of Clinical Oncology and College of American Pathologists joint review. *J Clin Oncol*. 2018;36(16):1631-1641.
54. Khagi Y, Goodman AM, Daniels GA, et al. Hypermutated circulating tumor DNA: correlation with response to checkpoint inhibitor-based immunotherapy. *Clin Cancer Res*. 2017;23(19):5729-5736.
55. Roy-Chowdhuri S, Stewart J. Preanalytic variables in cytology: lessons learned from next-generation sequencing—the MD Anderson experience. *Arch Pathol Lab Med*. 2016;140(11):1191-1199.
56. Jennings LJ, Arcila ME, Corless C, et al. Guidelines for validation of next-generation sequencing-based oncology panels: a joint consensus recommendation of the Association for Molecular Pathology and College of American Pathologists. *J Mol Diagn*. 2017;19(3):341-365.
57. Srinivasan M, Sedmak D, Jewell S. Effect of fixatives and tissue processing on the content and integrity of nucleic acids. *Am J Pathol*. 2002;161(6):1961-1971.
58. Einaga N, Yoshida A, Noda H, et al. Assessment of the quality of DNA from various formalin-fixed paraffin-embedded (FFPE) tissues and the use of this DNA for next-generation sequencing (NGS) with no artifactual mutation. *PLoS One*. 2017;12(5):e0176280.
59. Howat WJ, Wilson BA. Tissue fixation and the effect of molecular fixatives on downstream staining procedures. *Methods*. 2014;70(1):12-19.
60. Feliubadaló L, Tonda R, Gausachs M, et al. Benchmarking of whole exome sequencing and ad hoc designed panels for genetic testing of hereditary cancer. *Sci Rep*. 2017;7:37984.
61. Qiu P, Pang L, Arreaza G, et al. Data interoperability of whole exome sequencing (WES) based mutational burden estimates from different laboratories. *Int J Mol Sci*. 2016;17(5):651.
62. Baras AS, Stricker T. Characterization of total mutational burden in the GENIE cohort: small and large panels can provide TMB information but to varying degrees. *Cancer Res*. 2017;77(suppl 13): Abstract LB-105. Available at [http://cancerres.aacrjournals.org/content/77/13\\_Supplement/LB-105](http://cancerres.aacrjournals.org/content/77/13_Supplement/LB-105).
63. Garofalo A, Sholl L, Reardon B, et al. The impact of tumor profiling approaches and genomic data strategies for cancer precision medicine. *Genome Med*. 2016;8(1):79.
64. Lee C, Bae JS, Ryu GH, et al. A method to evaluate the quality of clinical gene-panel sequencing data for single-nucleotide variant detection. *J Mol Diagn*. 2017;19(5):651-658.
65. Gong J, Pan K, Fakhri M, Pal S, Salgia R. Value-based genomics. *Oncotarget*. 2018;9(21):15792-15815.
66. Ng SB, Turner EH, Robertson PD, et al. Targeted capture and massively parallel sequencing of twelve human exomes. *Nature*. 2009;461(7261):272-276.

67. Buchhalter I, Rempel E, Endris V, et al. Size matters: dissecting key parameters for panel-based tumor mutational burden (TMB) analysis. *Int J Cancer*. 2018;144:848-858. <https://doi.org/10.1002/ijc.31878>.
68. Allgäuer M, Budczies J, Christopoulos P, et al. Implementing tumor mutational burden (TMB) analysis in routine diagnostics—a primer for molecular pathologists and clinicians. *Transl Lung Cancer Res*. 2018;7(6):703-715.
69. Endris V, Buchhalter I, Allgauer M, et al. Measurement of tumor mutational burden (TMB) in routine molecular diagnostics: in-silico and real-life analysis of three larger gene panels. *Int J Cancer*. 2018. <https://doi.org/10.1002/ijc.32002>.
70. Singh RR, Patel KP, Routbort MJ, et al. Clinical validation of a next-generation sequencing screen for mutational hotspots in 46 cancer-related genes. *J Mol Diagn*. 2013;15(5):607-622.
71. Kroigard AB, Thomassen M, Laenholm AV, Kruse TA, Larsen MJ. Evaluation of nine somatic variant callers for detection of somatic mutations in exome and targeted deep sequencing data. *PLoS One*. 2016;11(3):e0151664.
72. Koeppel F, Blanchard S, Jovelet C, et al. Whole exome sequencing for determination of tumor mutation load in liquid biopsy from advanced cancer patients. *PLoS One*. 2017;12(11):e0188174.
73. Lauss M, Donia M, Harbst K, et al. Mutational and putative neoantigen load predict clinical benefit of adoptive T cell therapy in melanoma. *Nat Commun*. 2017;8(1):1738.
74. Gerstung M, Beisel C, Rechsteiner M, et al. Reliable detection of subclonal single-nucleotide variants in tumour cell populations. *Nat Commun*. 2012;3:811.
75. Campbell BB, Light N, Fabrizio D, et al. Comprehensive analysis of hypermutation in human cancer. *Cell*. 2017;171(5):1042-1056.
76. Rajendran BK, Deng CX. Characterization of potential driver mutations involved in human breast cancer by computational approaches. *Oncotarget*. 2017;8(30):50252-50272.
77. Frampton GM, Fichtenholtz A, Otto GA, et al. Development and validation of a clinical cancer genomic profiling test based on massively parallel DNA sequencing. *Nat Biotechnol*. 2013;31(11):1023-1031.
78. Thomas SJ, Snowden JA, Zeidler MP, Danson SJ. The role of JAK/STAT signalling in the pathogenesis, prognosis and treatment of solid tumours. *Br J Cancer*. 2015;113(3):365-371.

**How to cite this article:** Stenzinger A, Allen JD, Maas J, et al. Tumor mutational burden standardization initiatives: Recommendations for consistent tumor mutational burden assessment in clinical samples to guide immunotherapy treatment decisions. *Genes Chromosomes Cancer*. 2019;58:578–588. <https://doi.org/10.1002/gcc.22733>