illumina®

APPLICATION NOTE

Assessing *MTAP* copy number variation with TruSight™ Oncology 500 v2

Optimized spike-in protocol enables *MTAP* CNV evaluation

Fast, end-to-end research workflow delivers results in 3–4 days

Powerful DRAGEN™ analysis identifies *MTAP* CNVs

Introduction

Homozygous deletion of the *MTAP* gene, a type of copy number variation (CNV), has emerged as an important biomarker in various solid tumors.[1] The *MTAP* gene lies in the 9p21 region of chromosome 9 (Figure 1), adjacent to *CDKN2A*, an important tumor suppressor gene that is frequently altered in cancers. Loss of *MTAP* expression is reported in up to 30% of cholangiocarcinomas and esophageal cancers, 14% of gastric cancers, 60% of glioblastomas and head and neck squamous cell carcinomas, and 40% of nonsmall cell lung cancers.[1] *MTAP* loss increasingly informs enrollment in protein arginine methyltransferase 5 (PRMT5) and methionine adenosyl transferase 2A (MAT2A) inhibitor trials and is often assessed by low-throughput fluorescence-based assays, including immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH). Because these assays can be time and labor intensive, there is a growing need for high-throughput approaches to enable fold-change assessment of *MTAP* CNVs. Tumor fraction (TF)estimation is critical for identifying homozygous *MTAP* deletions.

TruSight Oncology 500 v2 is a next-generation sequencing (NGS) assay for detecting pan-cancer biomarkers, including DNA and RNA variant types, and genomic signatures, including microsatellite instability (MSI), and tumor mutational burden (TMB). This comprehensive research assay includes a fully integrated homologous recombination deficiency (HRD) panel. However, the assay currently does not include probes for the *MTAP* gene.

This application note describes a modified research workflow* in which an *MTAP* probe pool is spiked into the TruSight Oncology 500 v2 enrichment reaction to enable the fold-change assessment of *MTAP* copy number.

Key considerations

- This protocol uses the TruSight Oncology 500 v2 with HRD workflow and a commercially available *MTAP* probe pool used as an add-on to the TruSight Oncology 500 v2 DNA-only workflow
- Higher confidence results are obtained in formalin-fixed paraffin-embedded (FFPE) samples with tumor fraction between 23–90%
- Probe design prioritizes CNV assessment and does not support small DNA variant detection
- Fold-change thresholds for variant calling are workflow-specific and should be set based on orthogonally confirmed reference samples with *MTAP* copy number loss
- This protocol was tested on the TruSight Oncology 500 v2 DNA only workflow; though the DNA+RNA workflow was not tested in this study, impacts to RNA results are not expected

* This research workflow is not part of the standard TruSight Oncology 500 v2 product configuration and is not a validated or supported clinical assay. Performance characteristics described here are specific to the conditions tested in this study. Users should establish their own performance characteristics and variant calling thresholds.

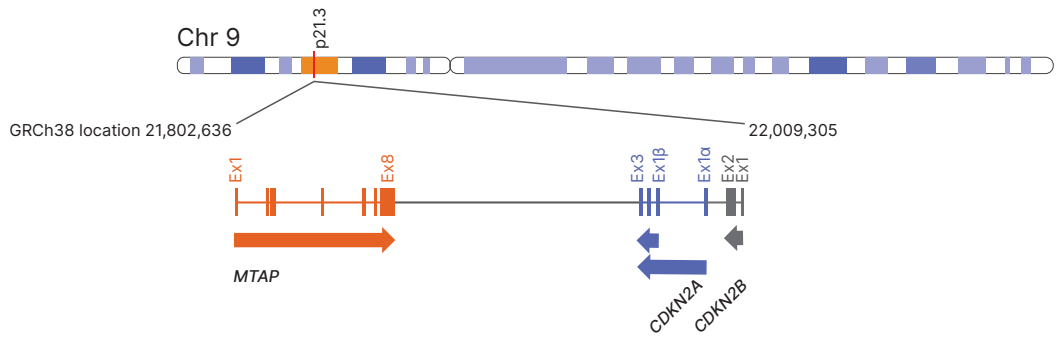

Figure 1: Location of *MTAP* and *CDKN2A* genes on chromosome 9
The *MTAP* gene is essential for purine salvage and polyamine metabolism. Due to its close proximity to *CDKN2A* (9p21.3), it is frequently homozygously deleted in gastrointestinal cancers, head and neck cancers, glioblastoma, pancreatic cancer, and nonsmall cell lung cancer.

Methods

The TruSight Oncology 500 v2 with HRD and *MTAP* probe spike-in workflow reports a fold-change value for the entire *MTAP* gene when assessing solid tumors for CNVs. An estimate of both tumor fraction and ploidy is essential when attempting to interpret fold changes for *MTAP* deletions. Combined enrichment with TruSight Oncology 500 v2 probes (OPD2 and OPD3)† enables both tumor fraction and ploidy estimations using the Myriad Genetics HRD algorithm. We recommend using the *MTAP* spike-in strategy detailed below in conjunction with the combined enrichment option of the DNA only workflow for TruSight Oncology 500 v2.

Library preparation and probe spike-in

The TruSight Oncology 500 v2 protocol was used to enable *MTAP* copy number variant assessment, using 30 ng of input for the DNA-only workflow, with the combined enrichment option (Figure 2).

† OPD2, oncology DNA probe pool 2; OPD3, oncology DNA probe pool 3 for HRD.

Preparation of the enrichment master mix, OPD2 + OPD3 HYB Master Mix (DNA + HRD only), in the 'Hybridize probes' step was modified to spike in *MTAP* probes (Table 1). Nominal probe pool concentration was determined based on the previously optimized OPD2 concentration in the enrichment reaction for TruSight Oncology 500 v2. The *MTAP* probe pool can be spiked into the enrichment master mix while minimizing excess volume. *MTAP* probes were brought to room temperature and mixed thoroughly before addition to the master mix.

Table 1: Modified enrichment master mix preparation with *MTAP* probe spike-in

Reagent	8 samples	16 samples	24 samples
NHB2	115.2 µl	230.4 µl	345.6 µl
OPD2	13.6 µl	27.2 µl	40.8 µl
OPD3	9.6 µl	19.2 µl	28.8 µl
MTAP probe pool	2 µl	4 µl	6 µl
EHB	23.2 µl	46.4 µl	69.6 µl

EHB, enrichment hybridization buffer; NHB2, hybridization buffer 2 + IDT NXT blockers; OPD2, oncology probe DNA 2; OPD3, oncology probe DNA 3.

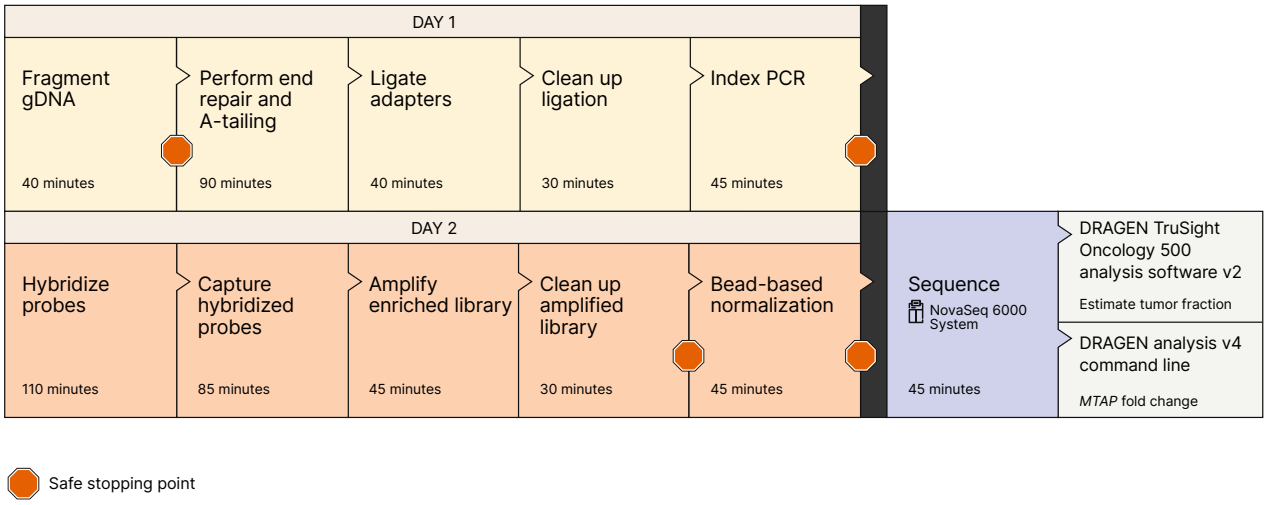

Figure 2: TruSight Oncology 500 v2 DNA-only workflow with combined enrichment and *MTAP* probe spike-in

Proof of principle study

Libraries were prepared following the TruSight Oncology 500 v2 protocol using the DNA-only workflow with 30 ng input DNA. Contrived reference control material (ODC4), cell line gDNA, and FFPE specimens were used as inputs for the assay (Table 2).

Studies were designed to assess the optimal spike-in concentration of *MTAP* probes, within-user repeatability of spike-in, performance of the TruSight Oncology 500 solid v2 assay with and without spike-in, and to build a panel of normals for CNV assessment. A commercially available cell line with a known *MTAP* homozygous deletion was characterized along with a small set of clinical research FFPE specimens.

Probe design and synthesis

The probe design prioritized uniform gene-level coverage for CNV assessment rather than complete exon coverage for small variant detection. Eighty-mer 5′ biotinylated probes were designed using a similar methodology to the TruSight Oncology 500 OPD2 panel. Probes were tiled across intronic and exonic regions of the *MTAP* gene. Probes that had a poorly predicted synthesis, were in low complexity (eg, homopolymer regions), or cross-reacted with probes in the OPD2 and OPD3 (HRD) panels were removed.

Additional probes were designed for two genes already present in the OPD2 panel (*ALK* and *ESR1*) to serve as internal comparators. These probes were designed for intronic regions so as not to interfere with existing OPD2 probes, enabling comparison of probe coverage between *MTAP* housekeeping probes and the analogous OPD2 probes as quality control for the spike-in. This probe pool has 196 unique probes: 146 *MTAP*, 25 *ALK*, and 25 *ESR1*. Probes were synthesized with a universal linker and purified via a streptavidin column to remove partial synthesis products from the probe pool. Probes were stored in 0.1× Tris-low EDTA (TLE) buffer.

MTAP probes are available commercially from Illumina (TruSight Oncology 500 *MTAP*, Catalog no. 20161477).

Spike-in titration

The working concentration of the *MTAP* probe pool ranged from 175 nM to 525 nM, corresponding to 7.5 pM to 22.5 pM per probe in the enrichment reaction. This resulted in five targets: 0.5×, 0.75×, 1× (same per probe concentration as OPD2), 1.25×, and 1.5×. Additionally, two control conditions were tested with the standard TruSight Oncology 500 v2 combined enrichment or with 2 µl of resuspension buffer (RSB) spiked in. Two master mixes were prepared by a single operator per dilution target and control condition, with two technical replicates of ODC4 each.

Table 2: Samples used in proof of principle studies

Experiment	Sample type	No. of samples	Sample details
Spike-in titration	TruSight Oncology DNA Control (ODC4) Catalog no. 20065041 (characterized *MTAP* retained)	24	Neat and buffer only n = 2 each 0.5×, 0.75×, 1.0×, 1.25×, 1.5× n = 4 each, 2 per master mix formulation
Spike-in reproducibility	TruSight Oncology DNA Control (ODC4) Catalog no. 20065041 (characterized *MTAP* retained)	20	0.5×, 0.75×, 1.0×, 1.25×, 1.5× n = 4 each, 2 per master mix formulation (same samples as above)
Concordance with and without spike-in	TruSight Oncology DNA Control (ODC4) Catalog no. 20065041 (characterized *MTAP* retained)	4	1.0× from above (n = 2) vs neat (n = 2) from above
Panel of normals	Benign adjacent FFPE samples	22	12 male 10 female
MTAP copy number loss control material	Contrived cell line-derived gDNA with *MTAP* copy number loss (SeraCare, Catalog no. MN 0710-1325)	18	0% (n = 4), 30% (n = 3), 60% (n = 3), 80% (n = 4), 100% (n = 4) tumor fraction
Clinical research samples	FFPE samples	13	*MTAP* LOH or copy number loss FFPE (n = 3), expected *MTAP* intact (n =10)

gDNA, genomic DNA; LOH, loss of heterozygosity.

Construction of baseline (panel of normals)

A baseline panel of normals was obtained using *MTAP* wild-type FFPE samples. Libraries were prepared using the spike-in modified master mix (Table 1) with the TruSight Oncology 500 v2 with HRD workflow and sequenced on the NovaSeq™ 6000 System. Data were characterized using the DRAGEN TruSight Oncology 500 analysis software v2.6.2.

For a sample to be included in the panel of normals, the following criteria had to be met:

- Passing all recommended DNA sample QC metrics
- No whole-gene CNVs identified

Twenty-two samples met these criteria and were included in the panel of normals, with 12 male and 10 female samples. Normal FFPE samples were derived from tissues adjacent to benign tumors from thyroid, liver, colorectal, small bowel, prostate, lung, uterus, and other cancer types.

Homozygous *MTAP* deletion cell line dilution

Cell line DNA with a known *MTAP* deletion and a matching wild-type cell line (SeraCare, Catalog no. MN 0710-1325) were serially diluted, ranging from expected tumor fraction of 100% (no *MTAP* coverage expected) to 0% (fully intact *MTAP*), and a target of 30%, 60%, and 80% TF. Four replicates at each dilution level were run through the TruSight Oncology 500 v2 DNA-only workflow with combined enrichment and *MTAP* spike-in. Data were analyzed using both the DRAGEN TruSight Oncology 500 analysis software and the DRAGEN Enrichment app with *MTAP* resources. Two replicates of each dilution level were orthogonally tested for *MTAP* CNV using digital droplet PCR (ddPCR).

Sequencing

Prepared libraries were sequenced on the NovaSeq 6000 System SP, S1, or S2 flow cells with 2 × 100 bp read lengths following the dilute and denature guide for the NovaSeq 6000 System. Users should follow the appropriate dilute and denature guide for the sequencing system they use.

Orthogonal confirmation of *MTAP* status using ddPCR

To orthogonally confirm fold changes observed in TruSight Oncology 500 v2 with *MTAP* spike-in, ddPCR was performed on the Bio-Rad QX200 system (Bio-Rad, Catalog no. 1864001) with cell line genomic DNA and clinical research FFPE samples. Validated CNV assays for *MTAP* (Bio-Rad, assay ID: dHsaCP1000039) on *FAM* and housekeeping gene *RPP30* (Bio-Rad, assay ID: dHsaCP1000485) on *HEX* were used. The Bio-Rad QuantaSoft Software (version 1.7.4.0917) was used to calculate copy number (CNV metric) and fold change (Ratio metric). One limitation of the copy number calculation for ddPCR is that it assumes the housekeeping gene *RPP30* is diploid.

The following formulas were used:

- Copy number = (number of *MTAP*+ droplets/number of *RPP30*+ droplets) × 2
- Fold change = (number of *MTAP*+ droplets/number of *RPP30*+ droplets)

Data analysis

DRAGEN TruSight Oncology 500 analysis software v2.6.2 was used without alteration for all non-*MTAP* results. For *MTAP*-specific results, the DRAGEN Enrichment app (v4.4.6) with the provided resource files was used.

MTAP analysis can also be performed by passing command line instructions with the same resource files.

Instructions to perform analysis are available here.

Minor differences were observed in the fold-change values obtained via the DRAGEN Enrichment app or command line methods using DRAGEN v4 and later (mean difference 0.046; 95% CI 0.03423–0.05831). The data presented in this application note were generated from the DRAGEN Enrichment app output.

Results

MTAP spike-in provides even coverage across the *MTAP* gene

The 1.0× dilution, targeting the same per probe concentration in the enrichment reaction as OPD2, produced the most similar coverage of *MTAP* probes compared to the OPD2 panel. Increasing *MTAP* probe pool concentration led to increased normalized coverage in the 1.25× and 1.5× test conditions (Figure 3). Non-normalized coverage was even across the *MTAP* gene, confirming that probe design and spike-in concentration were optimal for CNV assessment. Similar non-normalized coverage was observed between normal FFPE specimens and contrived ODC4 samples (Figure 4).

MTAP spike-in is reproducible

Minimal variability was observed between separate spike-in events at 1× dilution for probe coverage normalized by total PF reads (Figure 5). Within-operator variability was low; however, between-operator variability was not assessed.

Highly concordant TruSight Oncology 500 v2 results with and without *MTAP* spike-in

Key TruSight Oncology 500 v2 QC metrics were compared to confirm that the addition of the *MTAP* probe pool to the combined OPD2 + OPD3 enrichment does not alter the TruSight Oncology 500 v2 results. Though the performance of the DNA + RNA workflow was not assessed as part of this proof of principle study, no impacts to RNA results are expected.

Median exon coverage, percent (PCT) exon 50×, gene-scaled mean absolute deviation (MAD), and median bin count CNV target were comparable and highly concordant between the 1× *MTAP* spike-in and standard TruSight Oncology 500 v2 combined enrichment (Figure 6A).

CNV variant calling and small variant calling were highly concordant in ODC4 reference control material with and without *MTAP* spike-in (Figure 6B, Figure 6C). The results indicate that *MTAP* spike-in has no significant impact on variant calling for the TruSight Oncology 500 v2 assay.

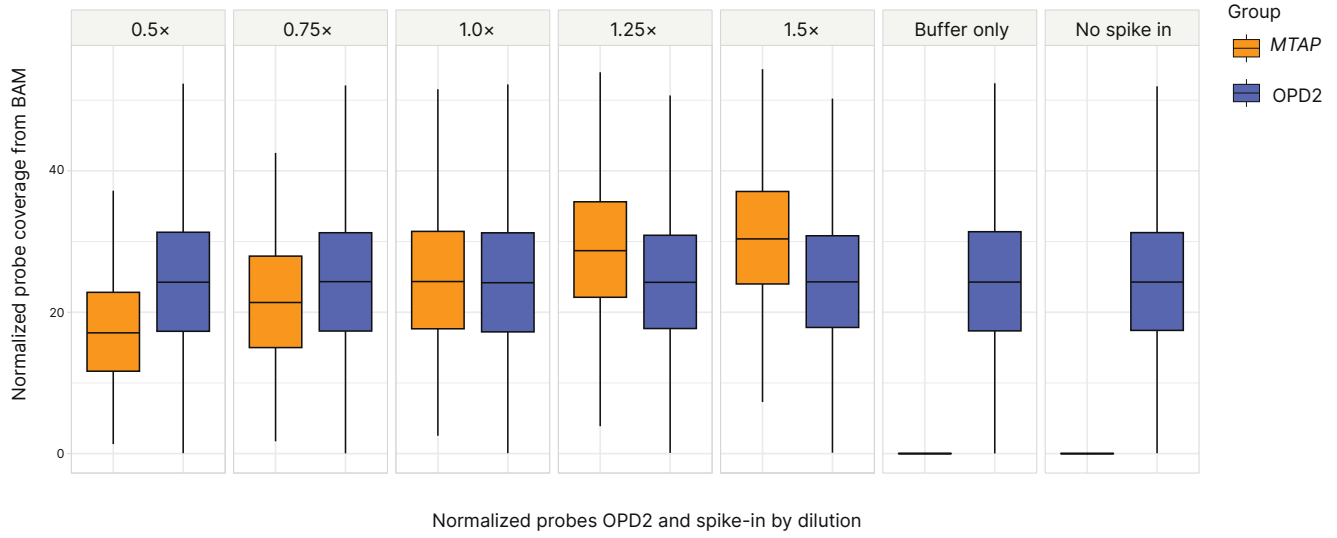

Figure 3: Normalized probe coverage across OPD2 and *MTAP* spike-in dilutions
Probe coverage mean normalized for reads in samples of *MTAP* probes was compared to OPD2 probes in a serial dilution from 0.5× to 1.5×. Neat samples without any spike-in and equal volume of buffer only were used as negative controls. Outliers were removed for easier visualization. There were no outliers in the *MTAP* probe pool however there were some high depth coverage regions in OPD2.
OPD2, oncology probe DNA 2.

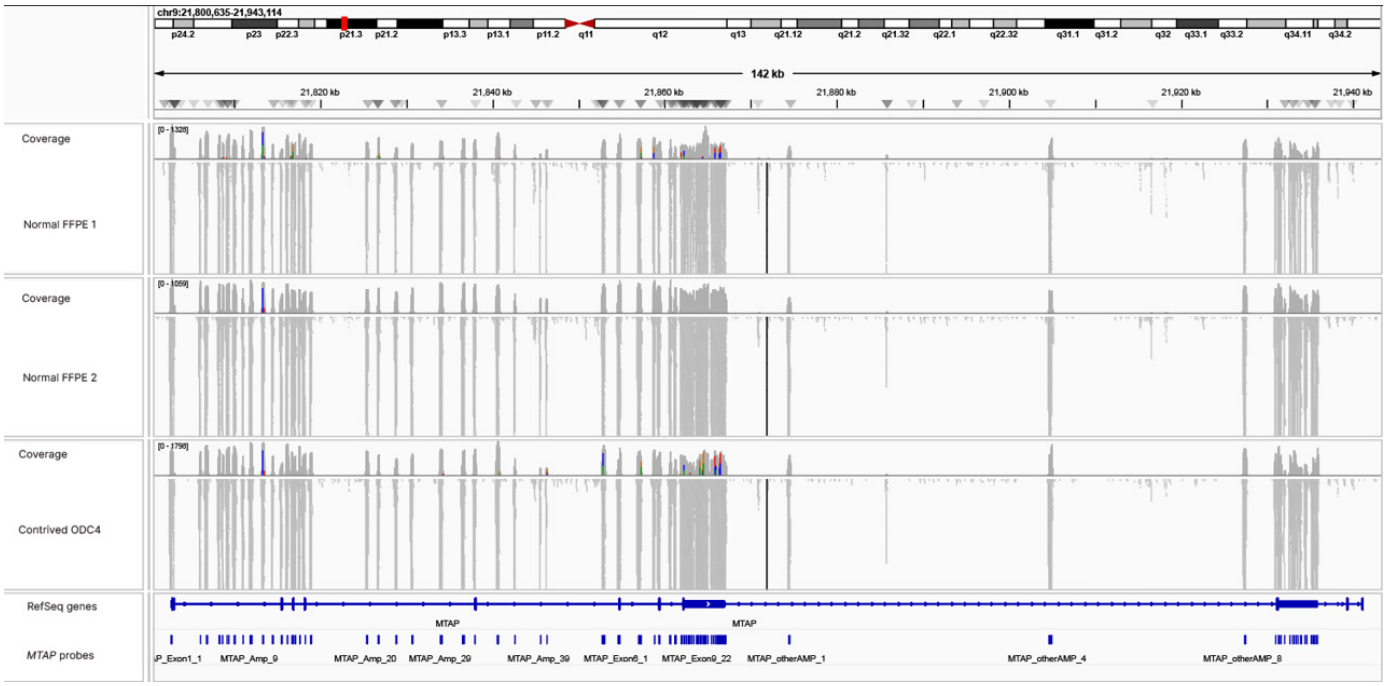

Figure 4: Comparable non-normalized *MTAP* gene coverage in normal FFPE and contrived control material ODC4 samples at 1.0×
FFPE, formalin-fixed paraffin-embedded; ODC4, oncology DNA control 4.

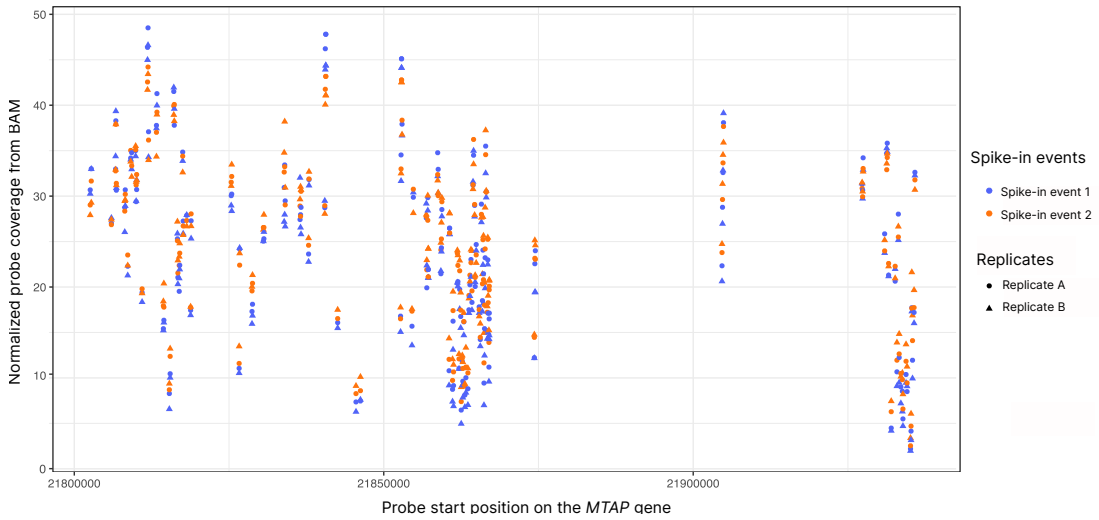

Figure 5: Normalized probe coverage across the *MTAP* gene between spike-in events
Low within-operator variability was observed between two independent master mix preparations at the 1.0× test condition.

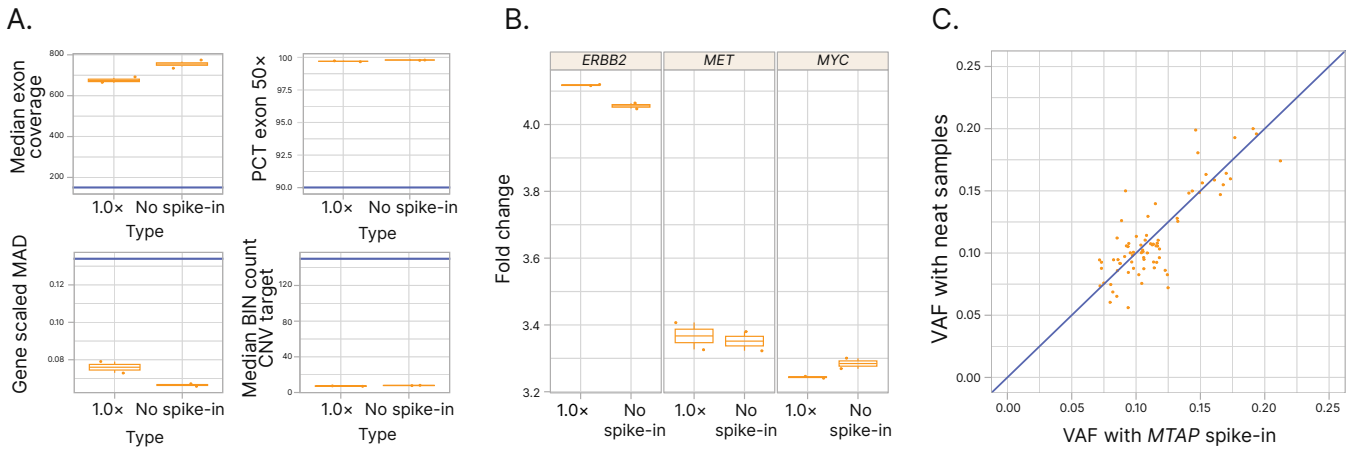

Figure 6: Concordance between TruSight Oncology 500 v2 sample QC metrics, CNVs, and small variant calling with and without *MTAP* spike-in libraries

(A)Key TruSight Oncology 500 v2 QC metrics, (B) fold change of called CNVs with and without *MTAP* spike-in, and (C) variant frequency (VF) concordance between neat and *MTAP* samples from truth variants.

Accurate results in cell line with known *MTAP* deletion

Fold-change results were highly concordant between the ddPCR and the DRAGEN Enrichment App in the cell line with a known *MTAP* homozygous deletion. A strong, inverse correlation (R^2 = 0.984) between the *MTAP* cell line tumor fraction and the fold change of the *MTAP* gene was observed (Figure 7), that is, fold change for a homozygous *MTAP* deletion decreases as tumor fraction increases. These results highlight the importance of tumor fraction estimation in the interpretation of *MTAP* fold change.

Characterization of FFPE samples with suspected *MTAP* deletion

Clinical research FFPE specimens were evaluated by both DRAGEN Enrichment App and ddPCR to determine *MTAP* status. Samples were filtered to only include those with tumor fraction between 23–90% and ddPCR to only include those with sufficient droplets for analysis. Three samples with suspected homozygous *MTAP* deletions were identified (Table 3).

Characterization of clinical research FFPE samples highlights the importance of tumor fraction and ploidy estimation for the interpretation of fold change from both *MTAP* probes and ddPCR. Tumor fraction and ploidy are reported in the TruSight Oncology 500 v2 with HRD combined variant output (GIS section). For confidence in estimations made by the HRD algorithm, the sample should pass all recommended pass/fail HRD-specific QC metrics (PCT target HRD 50× and excessive tumor fraction). Additionally, samples with lower tumor fraction and a higher amount of noise will have higher allele dosage ratios (ADR). A cutoff of < 0.85 indicates a good noise-to-signal ratio and a reliable tumor fraction estimation. Only samples with ADRs < 0.85 were included.

While there was general agreement between ddPCR and the DRAGEN Enrichment App, slight discrepancies between fold change values are expected due to different normalization methods used. ddPCR is normalized to a single housekeeping gene (*RPP30*) in this study. However, analysis by DRAGEN Enrichment App is normalized to a panel of normals. This method effectively normalizes the samples' segment mean for *MTAP* with respect to systematic noise observed within a population that has a similar wet lab workflow.

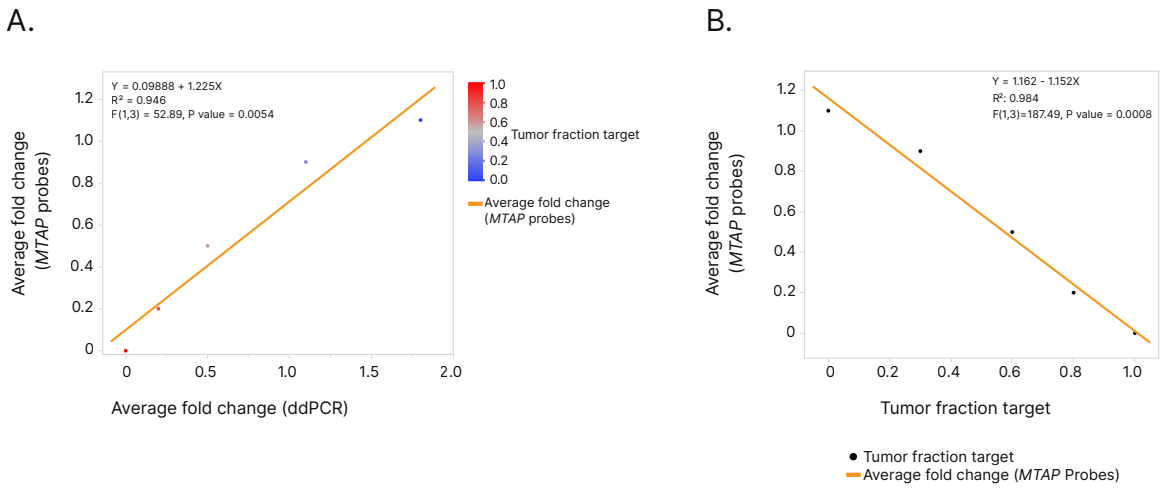

Figure 7: Relationship between average *MTAP* fold change results between (A) ddPCR and DRAGEN Enrichment and (B) *MTAP* probes and tumor fraction in a cell line with known homozygous *MTAP* deletion

Table 3: FFPE samples with MTAP deletion detected (as determined by < 0.5 ddPCR fold change)

Sample ID	Tumor type	Observed tumor fraction[a]	Ploidy[a]	Allele dose ratio[a]	MTAP fold change[b]	ddPCR average copy number[c]	ddPCR average fold change[d]
1985	Colorectal	0.37	3.00	0.306	0.817	0.953	0.478
3626	Lung	0.39	2.82	0.416	0.800	0.982	0.491
1594	Melanoma	0.44	3.78	0.185	0.819	0.8005	0.4005

a. TruSight Oncology 500 + HRD output.
b. From DRAGEN Enrichment output.
c. Bio-Rad QuantaSoft software 'CNV' output.
d. Bio-Rad QuantaSoft software 'Ratio' output.

Ten FFPE samples not included in the baseline and suspected to be *MTAP* wild-type were characterized with TruSight Oncology 500 v2 with HRD and the DRAGEN Enrichment App with *MTAP* resource files to evaluate the variability of fold change in normal samples. All samples passed TruSight Oncology 500 v2 QC metrics and had a tumor fraction of 0% and an ADR of 50. The mean *MTAP* fold change in the normal samples was 1.031 (min 0.885, max 1.325).

Interpreting *MTAP* fold change values

Fold-change values provide relative estimates of *MTAP* copy number and do not directly correspond to absolute copy-number states. *MTAP* results with a tumor fraction of 23–90% are high confidence. Fold change should be assessed in the context of sample quality, tumor purity, and baseline performance when establishing thresholds for CNVs.

For general interpretation of *MTAP* fold changes, the cell line data can be used in conjunction with the observed standard deviation of *MTAP* fold change in tested normal samples. The relationship between cell line *MTAP* fold change and the tumor fraction was established in the cell line dilution experiment. The graph displays a line of best fit and the line of fit offset with 1.5 standard deviations of the *MTAP* fold change from tested normal samples (Figure 8).

As an example, using sample ID 1985 from Table 3, which has a tumor fraction of 37%, and the model illustrated in Figure 8 on the blue line, an estimated upper limit for this tumor fraction can be generated:

Fold change estimated upper limit for 37% TF = 1.35 – (1.092 × 0.37) = 0.911

Because the sample has an observed fold change of 0.817, which is less than 0.911 (estimated upper limit for a given TF), this sample would be likely to have an *MTAP* deletion.

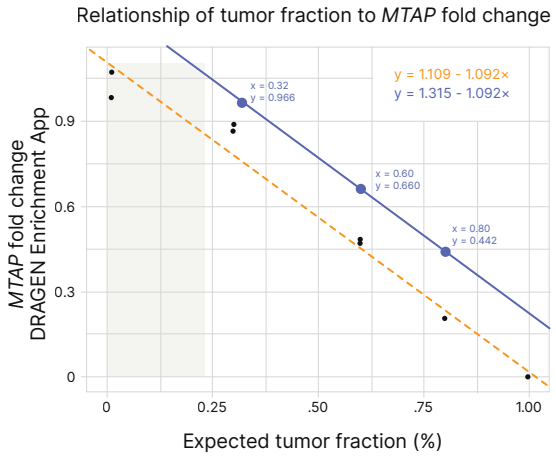

Figure 8: Relationship between tumor fraction and *MTAP* fold change

The grey zone shows range below 23% tumor fraction where accuracy is expected to be lower. The dashed orange line is the line of best fit for the cell line dilution experiment. The solid blue line has the same slope as the dashed orange line and adds an offset of 1.5× standard deviations of the *MTAP* fold change observed in the tested normal samples.

Relationship between copy number and fold change

Copy number can be estimated from fold change (FC) given estimates of tumor fraction (TF) and tumor ploidy (TP), not assuming diploidy, using the following equation:

$$\text{Copy number} = \frac{FC \times [\,TF \times TP + (1 - TF) \times 2] - (2 - 2 \times TF)}{TF}$$

This relationship, without accounting for biological or technical error, is shown in Figure 9. Two key trends are observed:

- As tumor ploidy increases, discrimination between hemizygous and homozygous deletions becomes less reliable
- As tumor fraction increases, regardless of ploidy, the separation between hemizygous and homozygous deletions increases, improving confidence in copy number classification

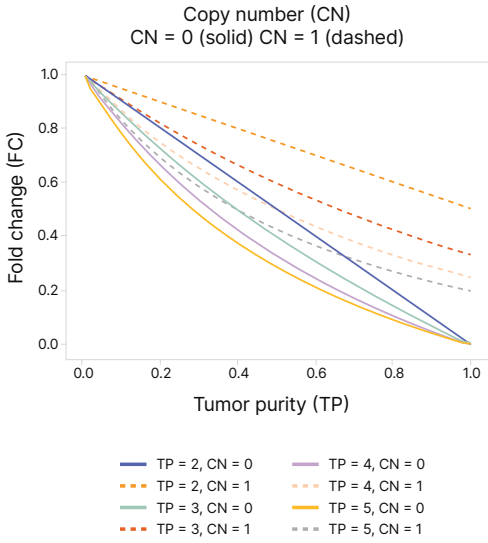

Figure 9: *In silico* model of relationship between tumor fraction and *MTAP* fold change

The solid lines represent homozygous (copy number 0) and the dashed lines represent hemizygous (copy number 1) *MTAP* deletions across varying tumor ploidy levels.

A limitation of copy number estimation is the inherent uncertainty associated with tumor fraction, whether derived computationally or by pathological assessment, as well as uncertainty in inferred tumor ploidy.

An additional consideration is cancer type. For example, glioblastoma, a cancer type with a relatively high prevalence of *MTAP* deletions, typically exhibits high tumor purity (> 75%).[2] As a result, tumor type–specific thresholds for calling *MTAP* deletions may be appropriate, rather than applying a single universal cutoff.

Summary

The proof of principle studies described in this application note demonstrate the performance of adding an *MTAP* probe pool to the TruSight Oncology 500 v2 with HRD enrichment assay for the assessment of CNV in the *MTAP* gene. The spike-in is repeatable across test events and shows good concordance with ddPCR. Additionally, these results underscore the importance of evaluating tumor fraction and ploidy for the assessment of *MTAP* fold change, regardless of assay type. Overall, these studies demonstrate the ability to identify *MTAP* homozygous deletions in samples with tumor fraction between 23–90%.

Learn more →

TruSight Oncology 500 v2

TruSight Oncology 500 HRD

DRAGEN Enrichment app

Reference

1. Clouser MC, Suh M, Movva N, et al. A systematic literature review of MTAP deletions in solid and hematologic cancers. *Cancer Treat Res Commun*. 2025; 44:100966. doi: 10.1016/j.ctarc.2025.100966.
2. Xiong Y, Xiong Z, Cao H, Li C, Wanggou S, Li X. Multi-dimensional omics characterization in glioblastoma identifies the purity-associated pattern and prognostic gene signatures. *Cancer Cell Int*. 2020;20:37. doi:10.1186/s12935-020-1116-3

illumina®

1.800.809.4566 toll-free (US) | +1.858.202.4566 tel
techsupport@illumina.com | www.illumina.com

M-GL-04207 v1.0