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Posted Date: 27 January 2025

doi: 10.20944/preprints202501.1965.v1

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*Article*

# A Novel Digital PCR Assay for Accurate Detection and Differentiation of Focal and Non-Focal Subtypes of *MET* Amplification in Lung Cancer

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**Abstract:** Mesenchymal–epithelial transition (*MET*) gene amplification is a critical biomarker in non-small cell lung cancer (NSCLC), significantly influencing treatment decisions and prognostic evaluations. However, current detection methods such as fluorescence in situ hybridization (FISH) and next-generation sequencing (NGS) have limitations in speed, cost, and specificity, particularly when distinguishing between focal MET amplification and MET polysomy. This study introduces a novel digital PCR (dPCR) assay designed not only to detect MET amplification but also to differentiate between its focal and non-focal subtypes. The assay was evaluated against established FISH and targeted NGS panels using 55 NSCLC samples with known MET amplification statuses (26 positive and 29 negative) confirmed by FISH and NGS. The dPCR assay demonstrated high sensitivity (96.0%) and specificity (96.7%), achieving 100% concordance with FISH in differentiating focal MET amplification from MET polysomy. Additionally, the assay exhibited excellent precision, accuracy, and linearity ($R^2 = 1.00$) in MET copy number quantification, surpassing NGS in diagnostic performance. Offering a robust, cost-effective, and efficient alternative to FISH, the dPCR assay significantly reduces turnaround time (3 hours versus 2 days) and provides a quantitative and objective method for MET amplification detection and subtype differentiation. This makes it suitable for clinical laboratories with limited molecular expertise. This study highlights the potential of the dPCR assay to complement existing molecular diagnostic techniques, delivering reliable and actionable results for MET-targeted therapy selection in NSCLC patients and thereby advancing precision oncology.

**Keywords:** *MET*; *MET* amplification; digital PCR; FISH; NGS; lung cancer; non-small cell lung cancer; NSCLC; focal *MET* amplification; non-focal amplification; *MET* polysomy

## 1. Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide, with non-small cell lung cancer accounting for approximately 85% of all lung cancer cases. (Shrivastava, 2018) (Bodén et al., 2023) While early-stage lung cancer patients have the best prognosis, the majority of patients are diagnosed with advanced or metastatic disease, resulting in a dismal 5-year survival rate of only 4%. (Weart et al., 2018) Precision medicine has emerged as a promising approach to improve outcomes for patients with lung cancer, with targeted therapies directed at specific genetic alterations playing a crucial role. The most well-known targetable gene alterations in lung cancer include mutations or amplifications in *EGFR* (10-15% of cases), *ALK* (2-7%), and *KRAS* (25-30%). (Choi & Chang, 2023)(Yu & Snyder, 2016) However, an increasing number of less common but still clinically relevant genetic

 doi:10.20944/preprints202501.1965.v1



targets, such as *MET* (Mesenchymal–epithelial transition) (3-7% of cases), are being identified and incorporated into treatment guidelines.(Choi & Chang, 2023)

*MET*, the proto-oncogene encoding the tyrosine kinase receptor for hepatocyte growth factor, has been found to be an important oncogenic driver in lung cancers, particularly in the adenocarcinoma subtype (Kumaki et al., 2023) (Yang et al., 2024) . *MET* can be altered through various mechanisms, including gene amplification, mutations, and exon 14 skipping. *MET* amplification is estimated to occur in approximately 3-5% of non-small cell lung cancer cases, while *MET* mutations and exon 14 skipping are observed in 3-4% and 3-5% of cases, respectively. (Korpanty et al., 2014) This genetic alteration can lead to aberrant activation of the *MET* signaling pathway, promoting cell proliferation, survival, and metastasis. *MET* amplification can be classified into two distinct subtypes: focal *MET* amplification, where the *MET* gene is selectively amplified, and non-focal amplification, which occurs due to polysomy of chromosomal 7, which is sometimes refer to as *MET* polysomy. Importantly, *MET* amplification has been associated with sensitivity to *MET*-targeted therapies. (Smit et al., 2021) (Ahn et al., 2021) The focal subtype of *MET* amplification, where the *MET* gene is selectively amplified, has been identified as a potential mechanism of resistance to EGFR tyrosine kinase inhibitors. This is particularly relevant, as patients with the focal *MET* amplification subtype may benefit from treatment with *MET*-targeted therapies that can overcome the resistance conferred by this genetic alteration. (Lai et al., 2019)(Corte et al., 2014) (Steen et al., 2019) Accurately detecting and differentiating between focal and non-focal *MET* amplification is critical for guiding appropriate treatment selection and management for these patients, yet it presents a significant challenge. (Kumaki et al., 2023) Recognizing the clinical significance of *MET* amplification, leading oncology guidelines, such as the National Comprehensive Cancer Network (NCCN) and the European Society for Medical Oncology (ESMO), have recommended routine testing for *MET* alterations in patients with lung cancer. (DiBonaventura et al., 2017)

The current common methods for *MET* amplification assessment in lung cancer, such as fluorescence in situ hybridization (FISH) and targeted next-generation sequencing panel (Targeted NGS panel), have limitations. FISH analysis for *MET* amplification has been hampered by a lack of consensus in interpretation, as well as technical challenges that necessitate scoring by medical experts, particularly in cases with high tumor heterogeneity and limited tissue availability. (Castiglione et al., 2018)(Hieggelke & Schultheis, 2020)(Tang et al., 2020) Targeted NGS panel, while more comprehensive for covering more actionable targets, can be costly and time-consuming, and lacking well defined cutoff and cannot differentiate between focal and non-focal *MET* amplification.(Wu et al., 2020)(Lee et al., 2021)(Guo et al., 2020). Digital PCR (dPCR) has emerged as a promising technique with high sensitivity and specificity for the absolute quantification of nucleic acids. Previous studies have demonstrated the feasibility of using droplet digital PCR (ddPCR) to detect *MET* amplification in Non-small-cell lung cancer (NSCLC) patients with EGFR-TKI resistance.(Oscorbin et al., 2022)(Heydt et al., 2019)(Fan et al., 2022) However, there has not been a study applying dPCR in the detection and differentiation of focal and non-focal MET amplification in lung cancer using Formalin fixed paraffin embedded (FFPE) tissue samples. Further research is needed to validate and optimize this approach not only for the accurate detection and also differentiation of focal and non-focal MET amplification in lung cancer.

To address these challenges, we have developed a novel dPCR assay at the molecular laboratory, Department of Pathology, Pamela Youde Nethersole Eastern Hospital, Hong Kong, that can accurately detect and discriminate between focal and non-focal *MET* amplification in lung cancer samples. The study will provide a streamlining workflow for the dPCR assay to make the assay more accessible and efficient, providing a simple user-friendly and cost-effective workflow for easy adoption for clinical laboratories, even for laboratories with limited expertise in molecular techniques.

## 2. Materials and Methods

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### 2.1. Study Design

This is a retrospective study involving 55 lung cancer patients between Jan 2023 and Oct 2024. Lung tissues were obtained from all the patients and targeted NGS panel results encompassing *MET* amplification status were obtained for all samples. There were in total 26 *MET* amplification positive samples and 29 *MET* amplification negative samples selected from the retrospective NGS results performed in the Molecular Laboratory, Pamela Youde Nethersole Eastern Hospital, Hong Kong. The samples selected were subsequently tested with our in-house dPCR assay and FISH to evaluate the performance of the dPCR assay in *MET* amplification detection and the differentiation of focal *MET* amplification and *MET* polysomy. In addition, commercial standard (Seraseq™ Lung & Brain CNV Mix) was used to evaluate the linearity and precision of the *MET* copy number (CN) quantification. This study was approved by the Central Institutional Review Board (CIRB) of the Hospital Authority Hong Kong (Approval No. HKECREC-2021-053).

### 2.2. DNA Isolation from FFPE Tissue Samples

Nucleic acid was extracted from FFPE tissue samples using the EZ2 AllPrep DNA/RNA FFPE Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. FFPE tissue sections of 10 μm thickness were cut and obtained, approximately totaling up to 2 $mm^3$ of tissue. The sections were deparaffinized with the Paraffin Removal Solution (PRS) supplied in the EZ2 AllPrep DNA/RNA FFPE Kit at 56°C for 3 min, followed by centrifugation at 20,000 x g for 2 min. The supernatant was removed, and the tissue pellet was resuspended by adding 150 μl of Buffer PKD and 10 μL of Proteinase K, then incubated at 56°C for 15 min at 500 rpm. The supernatant was transferred to a new 1.5 mL microcentrifuge tube for RNA preparation, and the tissue pellet was kept for DNA preparation. Subsequently, 180 μL of Buffer ATL and 40 μL of Proteinase K were added to the tissue pellet, which was then overlaid with 200 μl of PRS. mixture was loaded onto the EZ2 AllPrep DNA/RNA FFPE reagent cartridge, and the subsequent extraction was performed on the EZ2 Connect instrument (Qiagen, Hilden, Germany), with a 50 μL elution volume. The quantification of DNA extracts was performed following the manufacturer's recommendations, using the Qubit® dsDNA HS Assay Kit and the Qubit® Fluorometer (Thermo Fisher Scientific).

### 2.3. MET Amplification Detection Using FISH

FISH analysis was performed on selected FFPE tissue sections to assess *MET* gene amplification. The tissue sections were first deparaffinized, then underwent hybridization with a *MET*/CCP7 Dual Color FISH Probe (CytoTest Inc., Rockville, MD, catalog number CT-PAC014) according to the manufacturer's instructions. The *MET* gene is labeled with red fluorescence, while CCP7 is labeled with green fluorescence. All FISH results were scored by qualified pathologists. For scoring, a total of 50 representative tumor cells from random areas with homogeneous *MET* signal distribution were selected, and the non-overlapping nuclei were examined across multiple fields on each slide. The mean copy number (CN) of each probe was recorded for all the 50 cells, along with the percentage of cells exhibiting *MET* signal clusters and the percentage of cells with ≥ 5 copies of the *MET* signal. The *MET*/CCP7 ratio was calculated from the scores obtained from overall scoring. Based on previous reports, *MET* amplification was defined as having a *MET* CN ≥ 5 and a *MET*/CCP7 ratio ≥ 2.0. In contrast, *MET* polysomy was characterized as having a *MET* CN ≥ 5 combined with a *MET*/CCP7 ratio < 2.0. Cases that did not meet these criteria were considered as *MET* amplification negative. (Tong et al., 2016)(Lai et al., 2019)(Tanaka et al., 2012) Images of FISH including the different subtypes of *MET* amplification status are shown in Figure 1.

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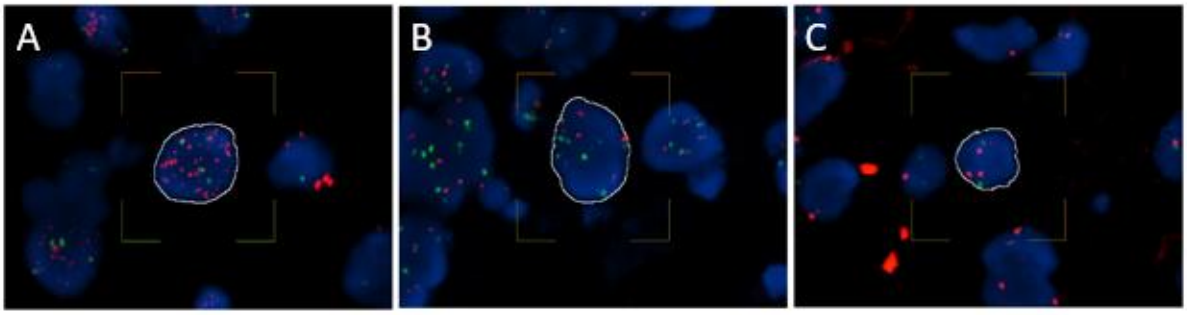


Images of FISH for different subtypes of *MET* amplification status were captured.
**A** Selected image of focal *MET* amplification.
**B** Selected image of *MET* polysomy.
**C** Selected image for *MET* amplification negative.

**Figure 1.** Images of FISH including the different subtypes of MET amplification status.

### *2.4. MET Amplification Detection Using NGS*

The extracted DNA samples were analyzed using the Thermo Fisher Scientific Oncomine Precision Assay GX (Thermo Fisher Scientific) on the Ion Torrent Genexus Integrated Sequencer (Thermo Fisher Scientific), following the manufacturer's instructions. This assay enables simultaneous detection of hotspot mutations across 50 cancer driver genes, which includes *MET* amplification. The library preparation was performed in the automated Ion Torrent Genexus Integrated Sequencer, utilizing 10 ng of FFPE-extracted DNA as the input. Sequencing was conducted on the Ion Torrent GX5 chip, and the sequencing data were mapped to the hg19 reference genome. Subsequently, the sequencing data were analyzed using the Ion Torrent software (Genexus software V.6.8.1.1 Thermo Fisher Scientific). *MET* amplification was called when the *MET* CN ≥ 5. All NGS results were reviewed and reported by qualified pathologists.

### *2.5. MET Amplification Detection Using dPCR in FFPE Samples*

DNA extracted from FFPE tissue samples was tested using our in-house developed dPCR assay for detecting *MET* amplification. The assay determines the CN of the *MET* gene and a reference gene locus (REF1), specifically the CELF2 gene locating on a different chromosome (chromosome 10), as well as the ratio between the *MET* gene and another reference gene locus (REF2), specifically the BRAF gene locating on the same chromosome (chromosome 7). Primer and probe sequences are listed in Table 1. This approach allows for the detection of *MET* amplification and also for differentiation between focal *MET* amplification and *MET* polysomy.

**Table 1.** Sequences of the primer and probe used in the dPCR assay.

| Target gene | Primer/Probe | Primer/Probe sequence |
|---|---|---|
| **MET** | MET_F | 5′-GACGGACCAGTCCTACATTGA-3′ |
| | MET_R | 5′-CTAGAGTTTCCCTTTGGACCG-3′ |
| | MET_P | 5′-FAM-CTTACCCCATTAAGTATGTCCATGCCTTTG-MGB-3′ |
| **CELF2 (REF1)** | REF1_F | 5′-AGAGGTTAACTTGGTGGCCT-3′ |
| | REF1_R | 5′-AAAACAAGCCGATGTAGTGGA-3′ |
| | REF1_P | 5′-HEX-AGAAGCCAGGAGAAGCACTTACTCCAA-MGB-3′ |
| **BRAF (REF2)** | REF2_F | 5′- AATAGAGTCCGAGGCGGG-3′ |
| | REF2_R | 5′- CCAATACCACAGGAAGAGGC-3′ |
| | REF2_P | 5′-HEX-GGATGATCCAGATGTTAGGGCAGTCTCT-MGB-3′ |

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The dPCR assay comprises two separate reaction mixes. Reaction 1 contains primers and probes targeting the *MET* gene and REF1. This reaction is used to determine the *MET* gene copy number within the tumor cell fraction in the sample. Since the tissue samples contain both tumor and normal cells, and normal cells are assumed to have a diploid status, we derived a formula to provide a more accurate calculation of the *MET* gene copy number in the tumor cell fraction. The wildtype copy number (CN) for both genes were determined through the multiplication of the overall CN of REF1 obtained from dPCR by the wildtype cell percentage (WT%), specifically calculated as 100% minus the pathologist-scored tumor percentage. Subsequently, the tumor fraction CN for both the *MET* and REF1 genes was derived by subtracting the overall CN of both genes obtained in dPCR from the calculated wildtype CN. It is postulated that each tumor cell maintains a normal diploid status for the REF1 gene, therefore the actual *MET* CN within an individual tumor cell was calculated by dividing the tumor fraction *MET* CN by the tumor fraction REF1 CN and multiplied by two. However, reaction 1 alone can only indicate the presence of *MET* gene amplification. It cannot differentiate between *MET* focal amplification and chromosome 7 polysomy.

Reaction 2 is the key for differentiating between *MET* focal amplification and non-focal *MET* amplification i.e. *MET* polysomy. It contains primers and probes targeting *MET* gene and REF2. The reaction is intended for determining the ratio between the *MET* gene and REF2 on the same chromosome. If the *MET* amplification is focal, only the *MET* CN would increase, while the REF2 CN would remain at 2, giving rise to a ratio ≥ 2. If the *MET* amplification is due to chromosome 7 polysomy, both the *MET* and REF2 (BRAF gene) copy numbers would increase proportionally, maintaining a ratio close to 1. Data analysis was performed to calculate the tumor fraction CN for both *MET* gene and REF2, subsequently the ratio between them. The overall CN from dPCR was determined for both the *MET* gene and REF2. The wildtype CN for both genes was calculated by multiplying the overall CN of REF2 obtained from dPCR by the WT%. Subsequently, the tumor fraction CN for both *MET* gene and REF2 was derived by subtracting the overall CN of both genes obtained from dPCR from the calculated wildtype CN. The tumor fraction *MET*/REF2 ratio was then calculated by dividing the tumor fraction *MET* CN by tumor fraction REF2 CN. Formula for result analysis for both reactions are summarized in Figure 2.

**(A) Formula for *MET* CN analysis in a single tumor cell from reaction 1**

$$\textit{MET}\ \text{CN in a single tumor cell} = \frac{\text{MET-(100\%-T\%) x REF1}}{\text{T\% x REF1}} \times 2$$

MET = Total dots of FAM signal in reaction 1
REF1 = Total dots of HEX signal in reaction 1
T% = Tumor percentage

**(B) Formula for *MET*/REF2 ratio analysis from reaction 2**

$$\textit{MET}/\text{REF2 ratio} = \frac{\text{MET-(100\%-T\%) x REF2}}{\text{T\% x REF2}}$$

MET = Total dots of FAM signal in reaction 2
REF2 = Total dots of HEX signal in reaction 2
T% = Tumor percentage

**Figure 2.** Formula for dPCR result analysis for both reaction 1 and reaction 2.

The analyzed results from both reactions are considered together for interpretation and summarized in Table 2. The *MET* amplification status is categorized into three subtypes: focal *MET* amplification, *MET* polysomy and *MET* amplification negative. Focal *MET* amplification is defined

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as having a tumor fraction *MET* CN ≥ 5 in reaction 1, and simultaneously a tumor fraction MET/BRAF ratio ≥ 2 in Reaction 2; *MET* polysomy is defined as having a tumor fraction *MET* CN ≥5 in reaction 1, but a tumor fraction MET/REF2 ratio < 2; *MET* amplification negative is defined as having a tumor fraction *MET* CN <5 in reaction 1.

**Table 2.** Result interpretation for the dPCR assay.

| Interpretation | *MET* copy number in reaction 1 | MET/BRAF ratio in reaction 2 |
|---|---|---|
| Focal *MET* amplification | ≥ 5 | ≥ 2 |
| *MET* polysomy | ≥ 5 | < 2 |
| *MET* amplification negative | <5 | N/A |

*2.6. Statistical Analysis*

The inter-run, intra-run precision, accuracy of CN calling and linearity for *MET* CN quantification were calculated based on the tested results obtained using commercial standard (Seraseq™ Lung & Brain CNV Mix). Linear Regression ($R^2$) was calculated for assessing the consistency and linearity of the data. While the performance of the dPCR assay in detecting *MET* amplification was evaluated by comparing the results to those obtained from the FISH and targeted NGS panel. Diagnostic performance (sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV)) of the dPCR assay were established with respective to the orthogonal tests including FISH and targeted NGS panel on the selected clinical samples.

## 3. Results

*3.1. Inter-Run/Intra-Run Precision, Accuracy of MET CN Calling and Linearity for MET CN Quantification Using Commercial Standard*

The inter-run and intra-run precision, as well as the accuracy of *MET* CN calling and linearity for *MET* CN quantification, were evaluated at three different *MET* CN levels: +2 CN, +6 CN, and +12 CN. These *MET* CN levels were determined using commercially available DNA standards with known *MET* gene CNs (Seraseq™ Lung & Brain CNV Mix), as well as a normal sample verified to confirm the absence of *MET* amplification. All levels were tested in triplicates across three separate runs. The inter-run and intra-run precision were calculated based on the coefficient of variation (CV%) of the triplicate measurements at each CN level. The mean CNs obtained from the dPCR assay were 2.21 (Range: 2.04-2.47; SD:0.19; CV:0.09; 95%CI) at the +2 CN level, 6.87 (Range: 6.83-6.95; SD:0.06; CV:0.01; 95%CI) at the +6 CN level, and 12.47 (Range: 12.19-12.85; SD:0.27; CV:0.02; 95%CI) at the +12 CN level. The mean CNs obtained for the three levels were in perfect correlation with the expected *MET* CNs, demonstrating the high precision and consistency of the *MET* CNs reported by the dPCR assay. While for accuracy, the observed *MET* CN by dPCR were 110.5%, 114.5%, and 103.9% of the expected values at the +2, +6, and +12 CN levels, respectively. Excellent linearity ($R^2$: 1.00) was also observed for the dPCR *MET* CN quantification. (Figure 3)

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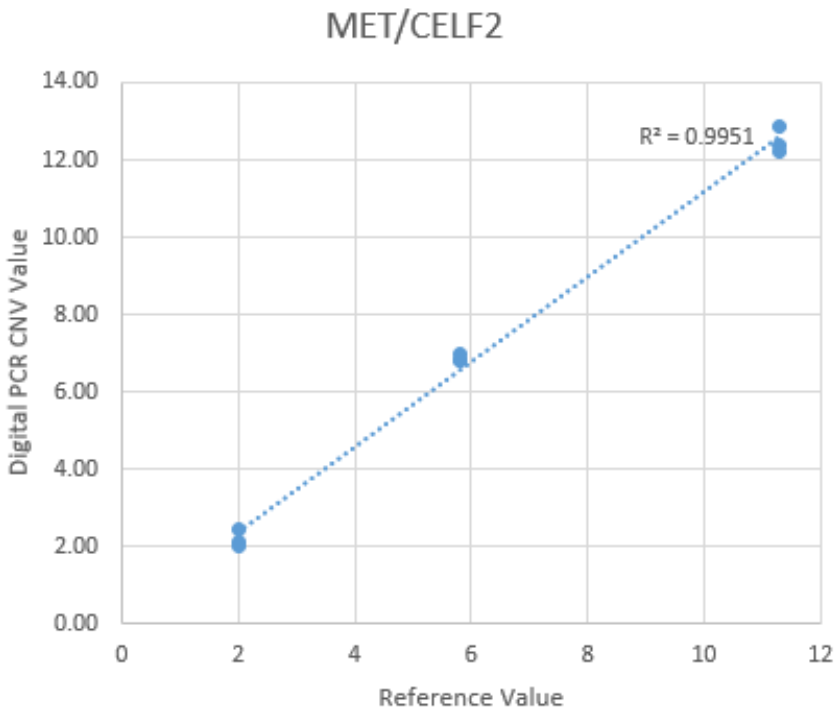


**Figure 3.** Correlation of dPCR *MET* CN quantification with reference.

*3.2. Overview of Result Obtained from the NGS, dPCR and FISH*

This study presents a comprehensive evaluation of the in-house dPCR for detection and classification of *MET* amplification against FISH and targeted NGS panel for FFPE tissues collected from 55 lung cancer patients. Overall results were summarized in Appendix A. Concordance of the positive results obtained by dPCR, FISH and NGS was shown in Figure 4.

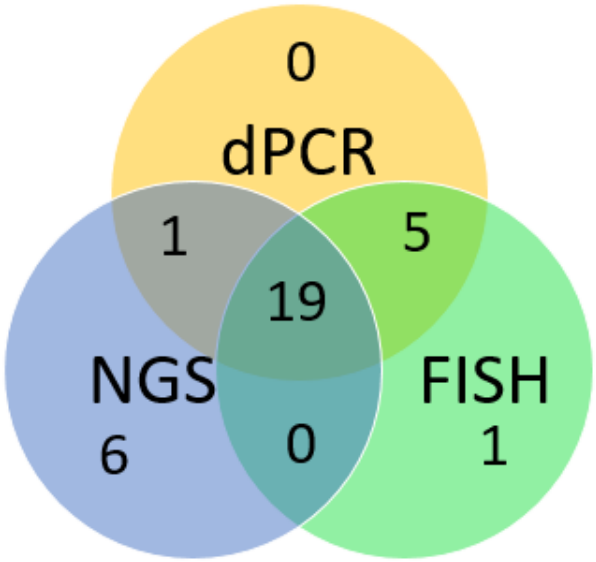


**Figure 4.** Concordance of the positive results obtained by dPCR, FISH and NGS.

*3.3. Assessing the Correlation Between dPCR and FISH on MET Amplification Detection and Differentiation*

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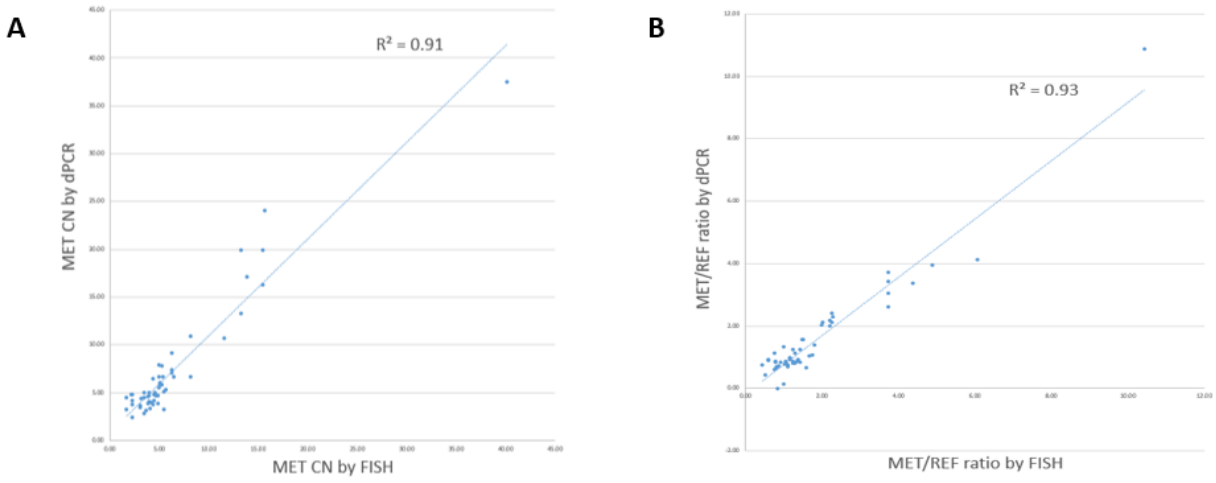
Among the 55 samples selected, 25 samples were detected with *MET* amplification by FISH, 15 of which were interpreted as focal *MET* amplification (*MET* CN ≥ 5 and *MET*/CEP7 ratio ≥2) and 10 samples as *MET* polysomy (*MET* CN ≥ 5 and *MET*/CEP7 ratio <2). The remaining 30 samples were FISH negative for *MET* amplification. Using FISH as gold standard result for assessing the performance of the dPCR assay, 24 out of 25 FISH-positive cases were detected as *MET* amplification positive by dPCR. For focal *MET* amplification, all of the 15 FISH positive cases were successfully detected and differentiated by dPCR, while for *MET* polysomy, 9 out of 10 FISH positive cases were correctly detected. For FISH negative cases, 29 out of 30 cases were correctly detected. Therefore, for *MET* amplification detection with reference to FISH results, the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were 96.0%, 96.7%, 96.0% and 96.7% respectively. For differentiation between focal *MET* amplification and *MET* polysomy, there was a 100% concordant rate between dPCR and FISH interpretation for all the dPCR positive cases. Also, for CN quantification and MET/REF2 ratio calculation, dPCR and FISH showed a good linear association (R=0.91 for CN quantification; R=0.93 for MET/REF2 ratio calculation) (Figure 5).




**A** shows the concordance between dPCR and FISH in *MET* CN detection.

**B** shows the concordance between dPCR and FISH in MET/REF ratio detection.

**Figure 5.** Concordance between dPCR and FISH.

*3.4. Compare the Performance Between dPCR and NGS on MET Amplification Detection*

In our current clinical practice, NGS is used for comprehensive gene panel testing of lung cancer patients. *MET* amplification is detected based on *MET* copy number variation (CNV), with a cutoff for NGS-positive *MET* amplification at *MET* CN greater than or equal to 5. However, NGS serves primarily as a screening tool, and any detected *MET* amplification requires subsequent confirmation by FISH. An assessment of NGS performance in detecting *MET* amplification, compared to FISH as the gold standard, revealed that out of 25 FISH-positive cases, NGS detected 19 as *MET* amplification positive, including 13 cases of focal *MET* amplification and 6 cases of *MET* polysomy. For the remaining 6 FISH-positive cases, NGS failed to detect the *MET* amplification. Among the 30 FISH-negative cases, 23 were also detected as *MET* amplification-negative by NGS, while 7 were detected as *MET* amplification positive. The sensitivity, specificity, positive predictive value, and negative predictive value of NGS were 76.0%, 76.7%, 73.1%, and 79.3%, with a linear regression $R^2$ value was 0.81, indicating a fair level of consistency. (Figure 6) In comparison, the dPCR assay developed in this study demonstrated significantly better performance in terms of sensitivity, specificity, PPV, and NPV. Performance indicators of dPCR and NGS with reference to FISH including sensitivity,

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specificity, positive predictive value, and negative predictive value are listed in Table 3 for comparison.

**Table 3.** Comparison between dPCR and NGS with reference to FISH for MET amplification detection.

| Method | Sensitivity | Specificity | PPV | NPV | Linear regression (R2) |
|---|---|---|---|---|---|
| **dPCR** | 96.0% | 96.7% | 96.0% | 96.7% | 0.91 |
| **NGS** | 76.0% | 76.7% | 73.1% | 79.3% | 0.81 |

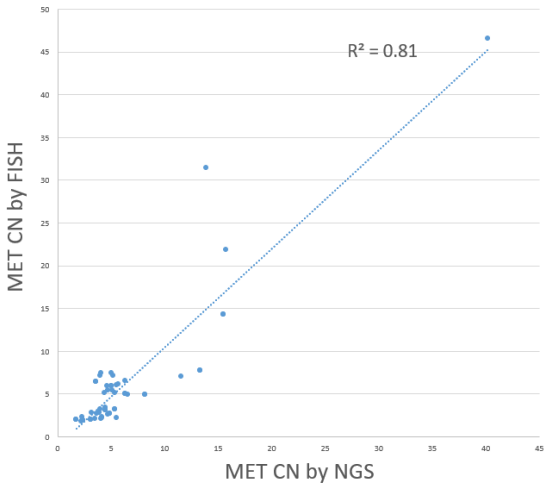


**Figure 6.** Concordance between NGS and FISH in MET CN detection.

Direct comparison of results of dPCR and NGS for *MET* amplification calling was performed. Comparison of the dPCR and NGS results on the 55 selected cases showed that 20 out of 26 NGS-positive cases were detected as *MET*-amplified by the dPCR assay, including 12 cases of focal *MET* amplification and 8 cases of *MET* polysomy. The remaining 6 NGS-positive cases were not detected as *MET*-amplified by the dPCR assay. Among the NGS-negative cases, 24 out of 29 were also tested as *MET* amplification-negative by the dPCR assay. The remaining 5 cases were detected as *MET* amplification-positive by dPCR. The positive percentage agreement, negative percentage agreement, and overall percentage agreement between dPCR and NGS were 79.9%, 82.8%, and 80%, respectively. Overall, dPCR and NGS showed a linear association. ($R^2$: 0.78). (Figure 7)

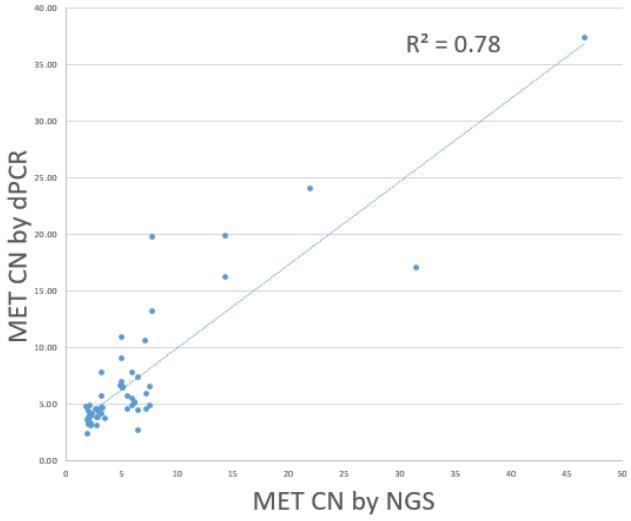


**Figure 7.** Concordance between dPCR and NGS in MET CN detection.

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## 4. Discussion

*MET* amplification has been associated with sensitivity to MET-targeted therapies. Growing evidence suggests that *MET* amplification is related to EGFR-TKI resistance and may contribute to acquired resistance in EGFR-mutated NSCLC patients treated with EGFR-TKIs. Accurately detecting and differentiating between focal and non-focal *MET* amplification is crucial for guiding appropriate treatment selection and management for these patients. In this study, we have developed and validated a novel digital PCR (dPCR) assay that enables the detection of *MET* amplification and the ability to differentiate between focal *MET* amplification and *MET* polysomy. Importantly, this study provides a comprehensive dataset consisting of results from NGS, dPCR, and FISH analyses, allowing for a thorough and objective performance comparison of these methodologies in detecting *MET* amplification.

The dPCR assay demonstrated high sensitivity and specificity of 96.0% and 96.7% respectively. It can also provide accurate *MET* CNs quantification comparable to the *MET* CN obtained from FISH, with a regression ($R^2$) of 1.0. The dPCR also achieved a 100% concordance rate with FISH for the differentiation of focal *MET* amplification and *MET* polysomy. The results demonstrate that the dPCR assay can offer performance comparable to the gold standard FISH, yet it possesses several advantages over FISH. Though FISH is the current gold standard for *MET* amplification detection, it is a labor-intensive process that requires specialized medical expertise, as the FISH scoring must be performed by qualified pathologists. Additionally, the FISH results interpretation can be subjective due to variations in scoring area selection, tumor heterogeneity, tissue section quality, and nucleic acid preservation. In contrast, the dPCR assay is faster, easier, simpler, less expensive, and more objective in quantifying gene CN comparing to FISH. The turn-around time (TAT) for the dPCR assay is around 3 hours versus at least 2 days for FISH.

In addition to FISH, NGS is another emerging method for lung cancer molecular diagnostics. NGS enables comprehensive gene panel testing, allowing it to assess a broad range of genetic targets for diagnostic and therapeutic purposes. While targeted NGS panels excel at detecting hotspot mutations at low variant allele frequencies (VAF), achieving accurate CNV calling, such as for *MET* amplification, can be challenging.(Singh et al., 2021)(Kumar et al., 2022) Factors like varied amplicon region coverage, amplification biases and artifacts, poor DNA quality from FFPE samples, and limitations of computational alignment tools can undermine the reliability of gene-level CNV assessments by NGS. As a result, detecting *MET* amplification through amplicon-based NGS approaches may not be as reliable as the more robust FISH and dPCR method. The findings of this study echo the above statements, the NGS-based assay showed a relatively lower sensitivity and specificity of 76.0% and 76.7% respectively in detecting *MET* amplification compared to the gold standard FISH method. The performance indicators, such as sensitivity, specificity, PPV and NPV, as well as the direct comparison between the dPCR and NGS results, demonstrated the superior performance of the dPCR assay over NGS in detecting *MET* amplification. Additionally, the dPCR assay can provide accurate differentiation between focal and non-focal amplification subtype, further enhancing its clinical utility, while NGS cannot. The dPCR assay developed in this study is also easy to adopt in the clinical laboratory, and it has the potential to serve as a reliable, accurate, and cost-effective alternative to FISH and a supplementary tool to NGS gene panel results for *MET* amplification detection and discrimination in lung cancer patients.

While the dPCR assay demonstrated good performance, there were two discordant cases between the dPCR assay and the FISH results. In one case, the dPCR assay found the sample to be positive, while FISH found it to be negative. In the other case, the dPCR assay found the sample to be negative, but FISH found it to be positive. For the dPCR-positive and FISH-negative case, the dPCR assay reported a tumor fraction *MET* CN of 6.43, while the *MET* CN obtained from FISH was 4.38, which is marginally below the cutoff of 5. For the dPCR-negative and FISH-positive case, the dPCR assay reported a tumor fraction *MET* CN of 3.16, while the *MET* CN obtained from FISH was 5.53 with a MET/CCP7 ratio at 1.11 (less than 2), which is marginally above the cutoff of 5 and hence interpreted as marginally *MET* polysomy. This discrepancy could be due to several reasons. The

 doi:10.20944/preprints202501.1965.v1



tumor cellularity across different tissue sections may vary, so the FISH result may not be fully representative of the entire tumor sample. In contrast, the dPCR assay is tested on DNA extracted from multiple tissue sections. Additionally, tumor heterogeneity is commonly observed among the tumor cells in a single tissue section, leading to varied *MET* CN counts from different tumor cells. The *MET* CN obtained from FISH is the average of 50 selected tumor cells, while dPCR quantifies the overall *MET* CN in the DNA samples representing the entire tumor sample. When considering the NGS result for the same cases, the *MET* CN detected for the dPCR-positive and FISH-negative case was 5.13, while for the dPCR-negative and FISH-positive case was 2.21, which was having aligned interpretation as the result of dPCR and reported as marginally positive and negative respectively. Therefore, the *MET* CN of this case is likely near the cutoff, and the discrepancy among the three testing methods is likely due to the differences in tumor cell abundance and genetic material heterogeneity. Despite these rare discordant cases, the overall performance of the dPCR assay was highly concordant with FISH, demonstrating its robust and reliable capability in detecting and differentiating *MET* amplification subtypes. The dPCR approach provides a more quantitative and objective assessment of *MET* copy numbers, which can complement the FISH results and help resolve challenging cases. These discordant cases also highlight the challenges in accurately detecting gene amplification, especially near the cutoff CN level, and the importance of utilizing complementary techniques like dPCR, FISH, and NGS to reach a reliable diagnosis. Therefore, FISH, while considered the gold standard, is not a perfect technique, and its results can be influenced by subjective interpretation, sample quality, and technical variations. The scoring of FISH signals by pathologists can be challenging, especially in samples with low-level amplification or tumor heterogeneity. This subjectivity in FISH interpretation may contribute to occasional discrepancies between FISH and other more quantitative techniques like dPCR. Besides, the dPCR assay developed in this study relies on the quantification of *MET* gene copies relative to a reference gene. While the assay demonstrated excellent linearity and precision, there may be rare instances where the reference gene copy number is also altered, leading to a skewed *MET* copy number assessment by dPCR.

There are several limitations to this study. The sample size is relatively small due to limited samples collected within the study period, impacting the ability to establish an accurate cutoff value for MET amplification calling. The current cutoff is based on general FISH standards. With a larger sample size, a more comprehensive comparison between dPCR, FISH, and NGS could be conducted, refining the cutoff value using Receiver Operating Characteristic (ROC) analysis. Based on our current data, adjusting the cutoff to a CN value between 4.5 to 5 may enhance concordance between dPCR and orthogonal tests. Additionally, the study did not assess the clinical utility of the dPCR assay in relation to treatment outcomes. Furthermore, exploring the assay's performance on alternative dPCR platforms could enhance its adaptability for varied clinical laboratory settings.

## 5. Conclusions

In conclusion, this study demonstrates the robustness and reliable performance of our in-house developed dPCR assay. The assay accurately detects and differentiates *MET* amplification subtypes, quantifies *MET* CN with high precision, and shows good concordance with the current gold standard FISH technique. It is seen to be a valuable complement to FISH and NGS testing for *MET* amplification assessment in clinical practice, offering advantages such as cost-effectiveness, faster turnaround time, and reduced sample requirements for a comprehensive molecular diagnostic for lung cancer patients in clinical use.

 doi:10.20944/preprints202501.1965.v1



# Appendix A

| | | NGS assay MET CN (Oncomine Comprehensive Assay | | FISH (Bioview) | | | | Reaction 1 Digital PCR | | | Reaction 2 Digital PCR | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample ID | Mean tumor % scored by two pathologist | MET CN | Pos/Neg | MET C | CCP7 C | MET/CCP7 Ratio | FISH Interpretat | Overall MET C | Overall REF1 (CELF2) C | Tumour fraction MET CN | Overall MET CN | Overall REF2 (BRAF) C | Tumor fraction MET/REF2 ratio |
| S01 | 30% | 5.96 | Pos | 5.01 | 2.76 | 1.82 | MET polysomy | 1971 | 1296 | 5.47 | 1718 | 1549 | 1.36 |
| S02 | 80% | 6.48 | Pos | 3.56 | 2.74 | 1.30 | Not detected | 15975 | 12380 | 2.73 | 11329 | 13542 | 0.80 |
| S03 | 30% | 1.77 | Neg | 2.18 | 2.34 | 0.93 | Not detected | 1782 | 1257 | 4.78 | 1529 | 1620 | 0.81 |
| S04 | 80% | 31.43 | Pos | 13.88 | 2.28 | 6.09 | Focal MET amp | 4198 | 598 | 17.05 | 4691 | 1341 | 4.12 |
| S05 | 90% | 5.47 | Pos | 4.7 | 4.01 | 1.17 | Not detected | 2608 | 1198 | 4.62 | 1765 | 1816 | 0.97 |
| S06 | 85% | 5.93 | Pos | 5 | 3.65 | 1.37 | MET polysomy | 2851 | 820 | 7.83 | 2365 | 2743 | 0.84 |
| S07 | 30% | 7.73 | Pos | 13.28 | 3.54 | 3.75 | Focal MET amp | 2414 | 900 | 13.21 | 2164 | 1342 | 3.04 |
| S08 | 60% | 7.08 | Pos | 11.58 | 2.36 | 4.91 | Focal MET amp | 1315 | 366 | 10.64 | 1278 | 463 | 3.93 |
| S09 | 30% | 1.93 | Neg | 2.36 | 2.74 | 0.86 | Not detected | 2102 | 1683 | 3.66 | 1390 | 1992 | -0.01 |
| S10 | 40% | 3.23 | Neg | 5.32 | 3.7 | 1.44 | MET polysomy | 298 | 170 | 5.76 | 358 | 387 | 0.81 |
| S11 | 50% | 4.9 | Neg | 6.52 | 5.2 | 1.25 | MET polysomy | 1805 | 835 | 6.65 | 1568 | 1736 | 0.81 |
| S12 | 40% | 2.28 | Neg | 4.18 | 5.18 | 0.81 | Not detected | 3601 | 2600 | 3.93 | 2586 | 2751 | 0.85 |
| S13 | 50% | 4.98 | Neg | 8.18 | 3.6 | 2.27 | Focal MET amp | 959 | 446 | 6.60 | 1035 | 667 | 2.10 |
| S14 | 90% | 6.1 | Pos | 5.48 | 3.24 | 1.69 | MET polysomy | 7432 | 3128 | 5.06 | 5629 | 5435 | 1.04 |
| S15 | 30% | 7.2 | Pos | 3.96 | 2.26 | 1.75 | Not detected | 731 | 529 | 4.55 | 778 | 765 | 1.06 |
| S16 | 20% | 46.55 | Pos | 40.21 | 3.85 | 10.44 | Focal MET amp | 18749 | 4128 | 37.42 | 7371 | 2478 | 10.87 |
| S17 | 30% | 5.13 | Pos | 5.38 | 2.69 | 2.00 | Focal MET amp | 958 | 568 | 6.58 | 889 | 682 | 2.01 |
| S18 | 30% | 6.13 | Pos | 5.7 | 5.32 | 1.07 | MET polysomy | 1533 | 1032 | 5.24 | 1860 | 1950 | 0.85 |
| S19 | 90% | 2.71 | Neg | 4.88 | 3.54 | 1.38 | Not detected | 1807 | 989 | 3.84 | 1839 | 2024 | 0.90 |
| S20 | 30% | 3.15 | Neg | 4.48 | 5.54 | 0.81 | Not detected | 5305 | 4017 | 4.14 | 2655 | 2949 | 0.67 |
| S21 | 70% | 2.06 | Neg | 3.1 | 5 | 0.62 | Not detected | 878 | 563 | 3.60 | 828 | 897 | 0.89 |
| S22 | 50% | 1.98 | Neg | 1.76 | 2.26 | 0.78 | Not detected | 629 | 480 | 3.24 | 715 | 901 | 0.59 |
| S23 | 35% | 21.91 | Pos | 15.74 | 3.58 | 4.40 | Focal MET amp | 6856 | 1412 | 24.03 | 5681 | 3123 | 3.34 |
| S24 | 90% | 6.51 | Pos | 6.28 | 4.98 | 1.26 | MET polysomy | 3690 | 1082 | 7.36 | 3093 | 3554 | 0.86 |
| S25 | 50% | 5.02 | Pos | 6.32 | 2.86 | 2.21 | Focal MET amp | 1892 | 840 | 7.01 | 1972 | 1324 | 1.98 |
| S26 | 80% | 7.48 | Pos | 4.04 | 2.68 | 1.51 | Not detected | 5482 | 2518 | 4.94 | 5116 | 3559 | 1.55 |
| S27 | 40% | 14.3 | Pos | 15.48 | 4.12 | 3.76 | Focal MET amp | 4091 | 1063 | 16.24 | 5332 | 2560 | 3.71 |
| S28 | 80% | 2.11 | Neg | 4.04 | 3.14 | 1.29 | Not detected | 1042 | 580 | 3.99 | 1088 | 1292 | 0.80 |
| S29 | 20% | 3.45 | Neg | 4.45 | 4.45 | 1.00 | Not detected | 1570 | 1341 | 3.71 | 1796 | 2181 | 0.12 |
| S30 | 45% | 2.87 | Neg | 3.94 | 3.6 | 1.09 | Not detected | 848 | 601 | 3.83 | 928 | 1035 | 0.77 |
| S31 | 15% | 2.6 | Neg | 4.71 | 2.97 | 1.59 | Not detected | 1583 | 1323 | 4.62 | 1602 | 1692 | 0.65 |
| S32 | 60% | 1.87 | Neg | 2.36 | 5.28 | 0.45 | Not detected | 643 | 575 | 2.39 | 683 | 814 | 0.73 |
| S33 | 30% | 2.35 | Neg | 2.28 | 4.26 | 0.54 | Not detected | 681 | 516 | 4.13 | 669 | 814 | 0.41 |
| S34 | 50% | 2.86 | Neg | 3.18 | 2.82 | 1.13 | Not detected | 734 | 462 | 4.35 | 768 | 880 | 0.75 |
| S35 | 70% | 3.27 | Neg | 4 | 3.96 | 1.01 | Not detected | 816 | 421 | 4.68 | 989 | 805 | 1.33 |
| S36 | 80% | 2.21 | Neg | 5.53 | 5 | 1.11 | MET polysomy | 636 | 435 | 3.16 | 605 | 810 | 0.68 |
| S37 | 60% | 2.27 | Neg | 4.12 | 4 | 1.03 | Not detected | 765 | 552 | 3.29 | 709 | 818 | 0.78 |
| S38 | 70% | 2.11 | Neg | 3.5 | 4.04 | 0.87 | Not detected | 355 | 175 | 4.94 | 284 | 356 | 0.71 |
| S39 | 60% | 2.75 | Neg | 3.68 | 4.62 | 0.80 | Not detected | 508 | 379 | 3.13 | 446 | 499 | 0.82 |
| S40 | 80% | 6.48 | Pos | 3.56 | 2.74 | 1.30 | Not detected | 2115 | 1062 | 4.48 | 1879 | 1735 | 1.10 |
| S41 | 90% | 5.47 | Pos | 5.1 | 4.36 | 1.17 | MET polysomy | 2087 | 778 | 5.74 | 1526 | 1638 | 0.92 |
| S42 | 85% | 5.93 | Pos | 4.6 | 3.65 | 1.26 | Not detected | 2888 | 1284 | 4.94 | 1633 | 1359 | 1.24 |
| S43 | 30% | 7.73 | Pos | 13.28 | 3.54 | 3.75 | Focal MET amp | 2096 | 571 | 19.81 | 1885 | 1094 | 3.41 |
| S44 | 30% | 1.93 | Neg | 2.36 | 2.74 | 0.86 | Not detected | 1378 | 980 | 4.71 | 1197 | 1326 | 0.68 |
| S45 | 40% | 3.23 | Neg | 5.32 | 3.7 | 1.44 | MET polysomy | 330 | 153 | 7.78 | 261 | 239 | 1.23 |
| S46 | 50% | 4.98 | Neg | 8.18 | 3.6 | 2.27 | Focal MET amp | 1358 | 421 | 10.90 | 1264 | 742 | 2.41 |
| S47 | 30% | 7.2 | Pos | 5.16 | 2.26 | 2.28 | Focal MET amp | 857 | 537 | 5.97 | 813 | 586 | 2.29 |
| S48 | 30% | 5.13 | Pos | 4.38 | 2.92 | 1.50 | Not detected | 867 | 521 | 6.43 | 714 | 615 | 1.54 |
| S49 | 90% | 2.71 | Neg | 4.88 | 3.54 | 1.38 | Not detected | 1824 | 838 | 4.61 | 1629 | 1854 | 0.87 |
| S50 | 30% | 3.15 | Neg | 4.48 | 5.54 | 0.81 | Not detected | 2284 | 1621 | 4.73 | 1915 | 2136 | 0.66 |
| S51 | 70% | 2.06 | Neg | 3.1 | 5 | 0.62 | Not detected | 981 | 657 | 3.41 | 791 | 852 | 0.90 |
| S52 | 50% | 1.98 | Neg | 1.76 | 2.26 | 0.78 | Not detected | 689 | 430 | 4.41 | 695 | 661 | 1.10 |
| S53 | 50% | 5.02 | Pos | 6.32 | 2.86 | 2.21 | Focal MET amp | 1787 | 647 | 9.05 | 1543 | 979 | 2.15 |
| S54 | 80% | 7.48 | Pos | 5.04 | 2.48 | 2.03 | Focal MET amp | 4823 | 1697 | 6.61 | 3542 | 1879 | 2.11 |
| S55 | 40% | 14.3 | Pos | 15.48 | 4.12 | 3.76 | Focal MET amp | 5518 | 1208 | 19.84 | 5137 | 3128 | 2.61 |

 doi:10.20944/preprints202501.1965.v1



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