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Comparison of *in situ* and extraction-based methods for the detection of *MET* amplifications in solid tumors

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ABSTRACT

In EGFR-treatment naive NSCLC patients, high-level MET amplification is detected in approximately 2–3% and is considered as adverse prognostic factor. Currently, clinical trials with two different inhibitors, capmatinib and tepotinib, are under way both defining different inclusion criteria regarding *MET* amplification from proven amplification only to defining an exact *MET* copy number. Here, 45 patient samples, including 10 samples without *MET* amplification, 5 samples showing a low-level *MET* amplification, 10 samples with an intermediate-level *MET* amplification, 10 samples having a high-level *MET* amplification by a MET/CEN7 ratio $\geq$2.0 and 10 samples showing a high-level *MET* amplification with GCN $\geq$6, were evaluated by MET FISH, MET IHC, a ddPCR copy number assay, a NanoString nCounter copy number assay and an amplicon-based parallel sequencing. The MET IHC had the best concordance with *MET* FISH followed by the NanoString copy number assay, the ddPCR copy number assay and the custom amplicon-based parallel sequencing assays. The concordance was higher in the high-level amplified cohorts than in the low- and intermediate-level amplified cohorts. In summary, currently extraction-based methods cannot replace the *MET* FISH for the detection of low-level, intermediate-level and high-level *MET* amplifications, as the number of false negative results is very high. Only for the detection of high-level amplified samples with a gene copy number $\geq$6 extraction-based methods are a reliable alternative.

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1. Introduction

In the past few years, several molecular alterations have been defined as "driver mutations" in non-small-cell lung cancer (NSCLC) providing novel treatment options. Besides approved drugs targeting pathologically activated receptors and signaling molecules (EGFR, ALK, ROS1, BRAF), other genetic alterations as mutations and gene amplifications of *KRAS, ERBB2*, and *MET*, and chromosomal translocations of *RET* and *NTRK* are currently under evaluation in clinical trials [1–3]. This requires the implementation of high-quality molecular diagnostics for the characterization of actionable targets and the control of emerging resistance mechanisms while at the same time taking into account tumor heterogeneity.

The *MET* gene located on chromosome 7 encodes the receptor tyrosine kinase hepatocyte growth factor receptor (HGFR) [4]. *MET* alterations include copy-number gains, as well as so-called *MET* exon 14 skipping mutations being single-nucleotide variants or insertions/deletions (indels) in the exon-intron junctions of exon 14 [5,6]. As this type of mutation confers increased sensitivity to MET inhibitors, clinical studies are under way investigating the role of MET-Inhibitors in *MET* exon 14 mutated tumors [7,8]. *MET* copy number gains are found in the presence and absence of *MET* exon 14 skipping mutations [6,9] and can also co-occur with other drivers like *EGFR* or *KRAS* mutations [10]. *MET* amplifications are described in treatment naïve NSCLC patients as well as patients who developed resistance under therapy with either first, second or third generation tyrosine kinase inhibitors (TKI) [11]. As for the above described *MET* mutations, different inhibitors are currently tested in clinical studies [12].

Currently, the standard method for the evaluation of *MET* copy number gains is fluorescence *in situ* hybridization (FISH) and for NSCLC complex signal patterns are described [10]. For different clinical studies different thresholds for the definition of amplification are used thus it is important to evaluate the most accurate technology to determine *MET* copy number prior to inclusion of patients. *In situ* based approaches like FISH and immunohistochemistry (IHC) are hampered by the observer-dependent evaluation but can take into account tumor heterogeneity manifested for example in focal amplification. Methods working with extracted nucleic acids like digital droplet PCR (ddPCR), parallel sequencing or the NanoString nCounter technology are easier to quantify with regard to gene copy number but do not allow morphological correlation.

In this study we compared two *in situ* based approaches for the analysis of *MET* amplification with three different assays based on extracted nucleic acids. We used a cohort of well-characterized formalin-fixed paraffin embedded (FFPE) samples from patients with NSCLC which was previously characterized by fluorescence *in situ* hybridization.

2. Material and methods

2.1. Tumor samples and nucleic acid extraction

The registry of the Institute of Pathology of the University Hospital Cologne, Germany, was retrospectively searched for non-small cell lung cancer cases. 45 patient samples were selected with *MET* FISH results, classified in the following 4 groups: 10 samples without *MET* amplification, 5 samples showing a low-level *MET* amplification with ≥40% of tumor cells showing ≥4 *MET* signals (FISH probes targeting *MET*), 10 samples with an intermediate-level *MET* amplification (≥50% of cells containing ≥5 *MET* signals), 10 samples having a high-level *MET* amplification by a MET/CEN7 ratio ≥2.0 and 10 samples showing a high-level *MET* amplification with gene copy numbers (GCN) per cell of ≥6, as previously defined [10]. High-level *MET* amplifications by MET/CEN7 ratio ≥2.0 or by gene copy numbers (GCN) per cell of ≥6 are mutually exclusive in this study.

All samples were routinely formalin-fixed and paraffin-embedded (FFPE) according to local practice. 10 μm thick sections were cut from the FFPE tissue blocks and deparaffinized. The tumor areas were macrodissected from unstained slides using a marked hematoxylin-eosin (H&E) stained slide as a reference.

Samples were digested overnight using proteinase K and DNA was isolated with the Maxwell 16 FFPE Plus Tissue LEV DNA Purification Kit (Promega, Mannheim, Germany) on the Maxwell 16 (Promega) following manufacturer's instructions.

2.2. Fluorescence in-situ hybridization

MET FISH analysis was performed on 4 μm thick FFPE slides cut and mounted on Microscope KP-PLUS slides (Klinipath, Duiven, The Netherlands). Tissue slides were hybridized overnight with the Zyto-Light SPEC MET/CEN7 Dual Color Probe (ZytoVision, Bremerhaven, Germany) as previously described [10]. Twenty contiguous tumor cell nuclei from three areas, resulting in a total of 60 nuclei, were individually evaluated. MET/CEN7 ratio, the percentage of tumor cells and the average MET copy number per cell were calculated FISH results were classified in the following 4 groups: High-level amplification was defined in tumors with a MET/CEN7 ratio ≥2.0 or an average *MET* GCN per cell of ≥6.0. Intermediate-level of GCN gain being defined as ≥50% of cells containing ≥5 *MET* signals. Low-level of GCN gain was defined as ≥40%

of tumor cells showing ≥4 *MET* signals. All other tumors were classified as negative.

2.3. Immunohistochemistry

MET IHC was performed on 1–2 μm thick FFPE slides cut and mounted on X-tra Adhesive Slides (Leica, Wetzlar, Germany) using a Ventana Benchmark Ultra automated immunostainer and the clone SP44 (Ventana Medical Systems, Tucson, AZ) as previously described [13]. Each sample was assessed using the following scoring criteria for staining intensity: 0 = no staining or <50% of tumor cells with any intensity; 1+ = ≥50% of tumor cells with weak staining but <50% with moderate or higher intensity, 2+ = ≥50% of tumor cells with moderate or higher staining but <50% with strong intensity and 3+ = ≥50% of tumor cells were stained with strong intensity. Score 2+ and 3+ were defined as positive and score 0 and 1+ as negative.

2.4. Droplet digital PCR (ddPCR) copy number assay

MET ddPCR was performed with the ddPCR probe assay designed for copy number variation analysis: *MET*, Human (BIO-RAD, Hercules, CA, USA). *EIF2C1* was used as non amplified reference gene. 10 ng of DNA were used for droplet generation and amplification according to the ddPCR CNV Assay protocol including a no template control (BIO-RAD). Amplified droplets were counted with the QX 200 droplet reader and analyzed with the QuantaSoft software (BIO-RAD). Based on previous validation studies, the gene was considered to be a single copy if the average copy number was below 1.4, two copies if between 1.5 and 2.4, three copies if between 2.5 and 3.4 and continued. A GNC ≥2.5 was considered positive, to minimize the number of false positive results.

2.5. NanoString copy number assay

All samples were analyzed for copy-number alterations of *MET* using the NanoString nCounter platform (NanoString Technologies, Seattle, WA, USA). Copy number analysis was performed as previously described [13,14] using 200–600 ng of genomic DNA. Based on the manufacturer's protocol, published literature [13,14] and validation studies the gene was considered to be a single copy if the average copy number was below 1.4, two copies if between 1.5 and 2.4, three copies if between 2.5 and 3.4 and continued. A GNC ≥2.5 was considered positive, to minimize the number of false positive results.

2.6. Custom amplicon-based parallel sequencing assays

For parallel sequencing, the DNA content was measured using a quantitative real-time PCR (qPCR) kit (GoTaq qPCR Master Mix; Promega). Isolated DNA was amplified with customized GeneRead DNAseq Targeted Panel V2 containing *MET* (LUN4, LUN5 – 5 amplicons covering *MET*) (Qiagen, Hilden, Germany) and the GeneRead DNAseq Panel PCR Kit V2 (Qiagen) or an Ion AmpliSeq Custom DNA Panel containing *MET* (LUN3 – 1 amplicon covering *MET*) (Thermo Fisher Scientific, Waltham, MA, USA) and the Ion AmpliSeq Library Kit 2.0 (ThermoFisher Scientific), following the manufacturer's instructions. Libraries were constructed using the Gene Read DNA Library I Core Kit and the Gene Read DNA I Amp Kit (Qiagen). After end-repair and adenylation, NEXTflex DNA Barcodes were ligated (Bio Scientific, Austin, TX, USA). Barcoded libraries were amplified, and final library products were quantified, diluted, and pooled in equal amounts. Finally, 12 pmol of the constructed libraries was sequenced on the MiSeq (Illumina, San Diego, CA, USA) with a MiSeq reagent kit V2 (300 cycles) (Illumina) following

the manufacturer's recommendations. Data were exported as FASTQ files.

For copy number detection, mapping was performed by BWA-MEM (BWA Ver. 0.7.17-r1188 [15]) to the reference genome hg19 using the default parameters. Read counts for samples and negative controls were created by GATK Ver. 4.1 CollectReadCounts. Subsequently the negative controls were used for creating the panel of normals (LUN3 n = 9, LUN4 n = 10, LUN5 n = 10) with GATK Ver. 4.1 CreateReadCountPanelOfNormals for each panel. Negative controls were selected from previous sequenced cases with a negative *MET* amplification status. The read counts of the samples were denoised using GATK Ver. 4.1 DenoiseReadCounts and the average log2 fold change of the *MET* amplicons was used for further comparison [16]. Samples with a log2 fold ≥0.5 were classified as *MET* amplified, to minimize the number of false positive results.

2.7. Statistics

Overall percentage agreement (OPA), negative percentage agreement (NPA) and positive percentage agreement (PPA) were calculated between FISH as reference to IHC, NanoString, ddPCR and parallel sequencing.

3. Results

45 samples analyzed by *MET* FISH were evaluated by MET IHC, the ddPCR copy number assay including *MET*, the NanoString copy number assay including *MET* and custom parallel sequencing assays. The 45 samples were divided into 4 cohorts: 10 samples without *MET* amplification, 5 samples showing a low-level *MET* amplification with ≥40% of tumor cells showing ≥4 *MET* signals, 10 samples with an intermediate-level *MET* amplification (≥50% of cells containing ≥15 *MET* signals), 10 samples having a high-level *MET* amplification by a MET/CEN7 ration ≥2.0 and 10 samples showing a high-level *MET* amplification with GCN per cell of ≥6.

3.1. Immunohistochemistry

All 45 cases were evaluated by MET IHC. In all cohorts MET IHC showed heterogeneous results in comparison to FISH (Table 1). In the cohort without *MET* amplification 8 cases (80%) had a score of 0–1+ and were classified as negative. Sample 7 had a score of 3+ and sample 1 a score of 2+, both samples were defined as positive. In the *MET* low-level amplified cohort all 5 samples had a positivity in MET IHC. Only samples 12–14 had scores of ≥2+ and were defined as positive (60%). In the *MET* intermediate-level amplified cohort 8 of 10 samples had a MET IHC score of 2+ to 3 + and were defined as positive (80%). Two samples, sample 20 and 23 had a MET IHC score of 0. In the high-level amplified cohort with a MET/CEN7 ratio ≥2 all samples had a MET IHC score of 2+ to 3+ (100%) and were defined as positive and in the high level amplified cohort with a *MET* GCN ≥6 9 of 10 samples had a MET IHC score of 2+ to 3+ (90%) and were defined as positive. Sample 36 was negative for MET IHC with a score of 0. In the *MET* high-level amplified cohorts the concordance between FISH and IHC was higher with 95% compared to the other cohorts were only 19 of 25 samples concurred (76%) (Table 2, Figs. 1–6).

Table 1

Results of 45 samples analyzed with *MET* FISH, MET IHC, ddPCR copy number assay, NanoString copy number assay and custom parallel sequencing assays. *MET* amplified/positive samples are highlighted in grey.

Sample	TCC (%)	MET FISH	MET IHC	NanoString (GCN)	ddPCR (GCN)	Parallel sequencing (log2)	NGS Panel
1	40	MET not amplified;Ratio 1.06, GCN 1.9	2+	MET not amplified (2.10)	MET amplified (2.60)	MET not amplified (0.11)	LUN3
2	70	MET not amplified; Ratio 1.43, GCN 1.72	0	MET not amplified (2.15)	MET not amplified (1.82)	MET not amplified (-0.18)	LUN3
3	40	MET not amplified;Ratio 1.63, GCN 2.15	0	MET not amplified (2.04)	MET not amplified (2.14)	MET not amplified (-0.03)	LUN4
4	70	MET not amplified;Ratio1.23, GCN 2.08	0	MET not amplified (1.99)	MET not amplified (2.12)	MET not amplified (-0.03)	LUN4
5	80	MET not amplified;Ratio 1.22, GCN 1.82	1+	MET not amplified (1.63)	MET not amplified (1.84)	MET not amplified (0.15)	LUN4
6	30	MET not amplified;Ratio 0.65, GCN 2.1	0	MET not amplified (2.47)	MET amplified (3.10)	MET not amplified (-0.51)	LUN3
7	90	MET not amplified;Ratio 1.17, GCN 2.07	3+	MET not amplified (2.34)	MET not amplified (2.39)	MET not amplified (-0.01)	LUN3
8	40	MET not amplified;Ratio 1.27, GCN 2.42	0	MET amplified (2.81)	MET not amplified (2.22)	MET not amplified (0.32)	LUN4
9	80	MET not amplified;Ratio 1.08, GCN 2.12	1+	MET not amplified (1.94)	MET not amplified (2.35)	MET not amplified (-0.09)	LUN5
10	40	MET not amplified;Ratio 0.99, GCN 1.92	1+	MET not amplified (2.00)	MET not amplified (2.29)	MET not amplified (-0.31)	LUN4
11	80	MET amplified; Ratio 1.28; 40%≥4; GCN 3.2, low-level	1+	MET amplified (2.91)	MET amplified (2.61)	MET not amplified (0.40)	LUN4
12	80	MET amplified.Ratio 1.87;41.67%≥4; GCN 3.58, low-level	2+	MET amplified (3.04)	MET amplified (3.80)	MET not amplified (0.24)	LUN3
13	20	MET amplified;Ratio 1.49;48.33%≥4; GCN 4.48, low-level	3+	MET not amplified (2.35)	MET amplified (2.53)	MET not amplified (0.30)	LUN4
14	40	MET amplified;Ratio 1.09;43.33%≥4; GCN 3.55, low-level	2+	MET not amplified (1.92)	MET not amplified (2.01)	MET not amplified (0.07)	LUN4
15	60	MET amplified;Ratio 1.59;40.00%≥4; GCN 3.02, low-level	1+	MET not amplified (2.33)	MET amplified (2.67)	MET not amplified (0.30)	LUN4
16	80	MET amplified;Ratio 1.22;63.00%≥5; GCN 5.73, intermediate-level	3+	MET amplified (4.89)	MET amplified (4.37)	MET amplified (1.45)	LUN3
17	50	MET amplified;Ratio 1.01;51.67%≥5; GCN 4.63, intermediate-level	2+	MET amplified (3.11)	MET amplified (3.35)	MET not amplified (0.40)	LUN3

18	50	MET amplified;Ratio 1.83;56.67%≥5; GCN 5.42, intermediate-level	3+	MET amplified (4.06)	MET amplified (4.05)	MET amplified (0.75)	LUN4
19	60	MET amplified;Ratio 1.25;61.67%≥5; GCN: 5.35, intermediate-level	2+	MET not amplified (2.09)	MET not amplified (2.48)	MET not amplified (0.36)	LUN4
20	90	MET amplified;Ratio 1.24;56.67%≥5; GCN 5.77, intermediate-level	0	MET not amplified (1.95)	MET not amplified (2.19)	MET not amplified (0.11)	LUN3
21	30	MET amplified;Ratio 1.83;71.67%≥5; GCN 5.33, intermediate-level	3+	MET not amplified (2.18)	MET not amplified (2.25)	MET not amplified (0.43)	LUN4
22	80	MET amplified;Ratio 1.18;51.67%≥5; GCN 4.90, intermediate-level	2+	MET amplified (3.33)	MET amplified (3.91)	MET amplified (0.54)	LUN4
23	90	MET amplified;Ratio 1.16;53.33%≥5; GCN 5.08, intermediate-level	0	MET not amplified (2.32)	MET not amplified (1.41)	MET not amplified (0.20)	LUN4
24	40	MET amplified;Ratio 1.23;51.67%≥5; GCN 4.68, intermediate-level	2+	MET not amplified (2.21)	MET not amplified (2.09)	MET not amplified (0.05)	LUN4
25	95	MET amplified;Ratio 1.95;56.67%≥5; GCN 5.53, intermediate-level	3+	MET amplified (3.28)	MET amplified (4.01)	MET amplified (1.07)	LUN4
26	50	MET amplified;Ratio 2.48; GCN 4.13, high-level	3+	MET not amplified (2.24)	MET not amplified (2.08)	MET not amplified (0.04)	LUN4
27	60	MET amplified; Ratio 2.23; GCN 3.45, high-level	3+	MET not amplified (2.42)	MET not amplified (2.35)	MET not amplified (0.20)	LUN4
28	40	MET amplified; Ratio 2.05; GCN 4.37, high-level	3+	MET amplified (2.67)	MET not amplified (2.03)	MET not amplified (-0.03)	LUN4
29	50	MET amplified;Ratio 3.90; GCN 5.87, high-level	3+	MET amplified (5.43)	MET amplified (5.43)	MET amplified (1.00)	LUN4
30	25	MET amplified; Ratio 2.13; GCN 4.18, high-level	3+	MET not amplified (2.20)	MET not amplified (1.99)	MET not amplified (0.41)	LUN4
31	60	MET amplified;Ratio 2.33; GCN 4.35, high-level	3+	MET amplified (3.49)	MET amplified (3.71)	MET amplified (0.69)	LUN4
32	60	MET amplified;Ratio 2.77; GCN 4.43, high-level	3+	MET amplified (2.73)	MET not amplified (2.38)	MET not amplified (0.36)	LUN4
33	70	MET amplified;Ratio 2.18; GCN 4.35, high-level	3+	MET amplified (4.47)	MET amplified (5.20)	MET amplified (0.59)	LUN5
34	30	MET amplified;Ratio 2.26; GCN 4.67, high-level	2+	MET amplified (2.84)	MET amplified (2.97)	MET not amplified (0.18)	LUN5
35	100	MET amplified;Ratio 2.41; GCN 4.10, high-level	2+	MET amplified (3.38)	MET amplified (3.45)	MET amplified (0.75)	LUN4
36	90	MET amplified;Ratio 1.11; GCN 7.33; high-level	0	MET amplified (2.70)	MET amplified (3.08)	MET not amplified (0.15)	LUN4
37	70	MET amplified;Ratio 1.55; GCN 7.50; high-level	3+	MET amplified (4.47)	MET amplified (5.08)	MET amplified (0.64)	LUN4
38	40	MET amplified;Ratio 1.50; GCN 6.87; high-level	3+	MET amplified (2.53)	MET amplified (2.54)	MET amplified (0.37)	LUN4
39	40	MET amplified;Ratio 0.89; GCN 6.60; high-level	3+	MET amplified (2.80)	MET not amplified (1.91)	MET amplified (0.81)	LUN4
40	30	MET amplified;Ratio 1.43; GCN 6.97; high-level	3+	MET amplified (2.72)	MET not amplified (1.85)	MET not amplified (0.00)	LUN4
41	30	MET amplified;Ratio 1.82; GCN 6.38; high-level	2+	MET not amplified (2.24)	MET amplified (2.00)	MET not amplified (-0.08)	LUN5
42	50	MET amplified;Ratio 1.69 ; GCN 10.82; high-level	3+	MET amplified (2.86)	MET amplified (2.79)	MET not amplified (0.34)	LUN5
43	30	MET amplified;Ratio 1.59; GCN 22.80; high-level	3+	MET amplified (5.16)	MET not amplified (1.43)	MET amplified (0.70)	LUN5
44	70	MET amplified;Ratio 1.52; GCN 6.17; high-level	2+	MET amplified (3.48)	MET amplified (3.23)	MET amplified (0.57)	LUN5
45	50	MET amplified;Ratio 1.25; GCN 29.22; high-level	3+	MET amplified (25.00)	MET amplified (29.20)	MET amplified (2.55)	LUN5

TCC: Tumor cell content, GCN: Gene copy number
Tumor cell content, GCN: Gene copy number.

Table 2
Concordance between MET FISH and MET IHC, NanoString copy number assay, ddPCR copy number assay and custom amplicon-based parallel sequencing assays.

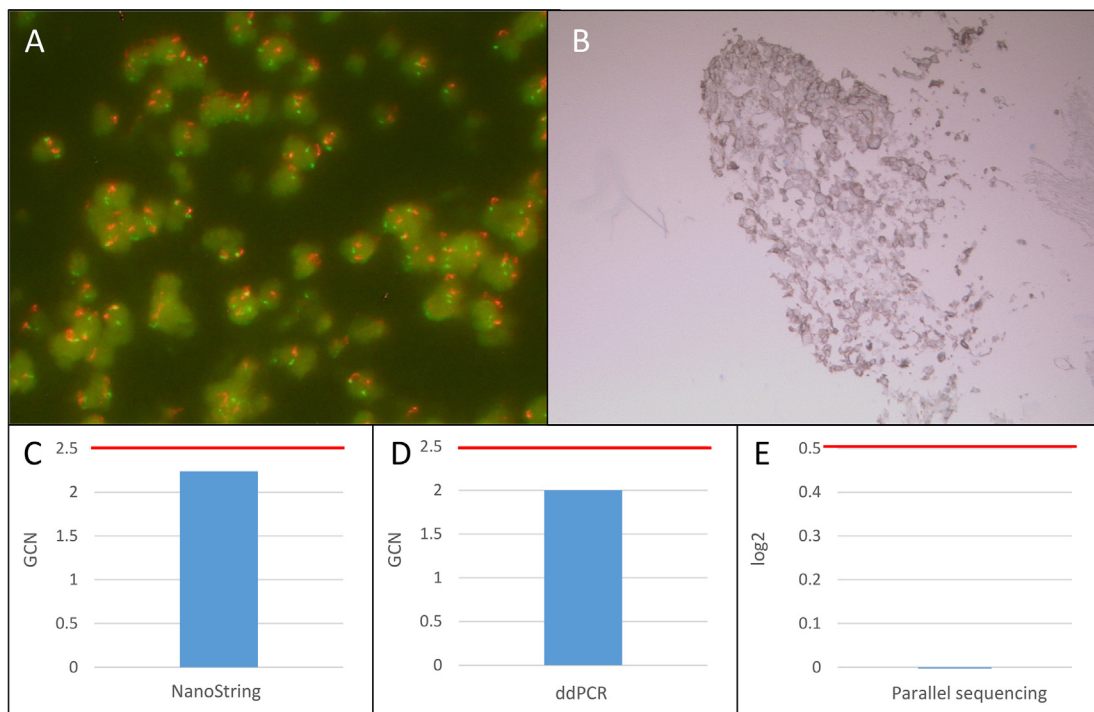|	MET FISH				
	MET without amplification	MET low-level amplification	MET intermediate-level amplification	MET/CEN7 ratio ≥2high-level amplification	MET GCN ≥6 high-level amplification
MET IHC	8/10 (80%)	3/5 (60%)	8/10 (80%)	10/10 (100%)	9/10 (90%)
ddPCR	8/10 (80%)	4/5 (80%)	5/10 (50%)	5/10 (50%)	6/10 (60%)
NanoString	9/10 (90%)	2/5 (40%)	5/10 (50%)	7/10 (70%)	9/10 (90%)
Parallel sequencing	10/10 (100%)	0/5 (0%)	4/10 (40%)	4/10 (40%)	5/10 (50%)

3.2. Droplet digital PCR copy number assay

The results of the ddPCR copy number assay are depicted in Table 1. For all samples the ddPCR copy number assay was performed. In the cohort without MET amplification 8 of 10 (80%) sam-ples had a copy number below 2.5 and were classified as negative. Samples 1 and 6 were positive by the ddPCR copy number assay. In the MET low-level amplified cohort 4 of 5 samples (80%) had a GCN higher than 2.5 and in the MET intermediate-level amplified cohort 5 of 10 samples (50%) had a GCN higher than 2.5 and were classi-
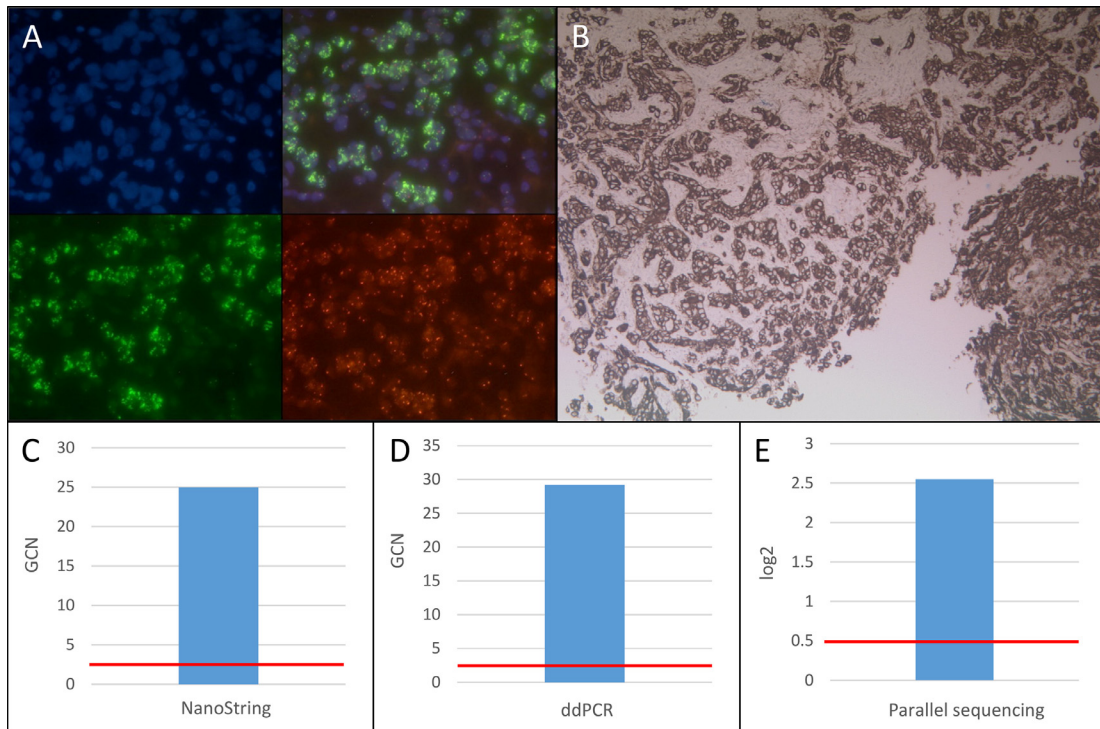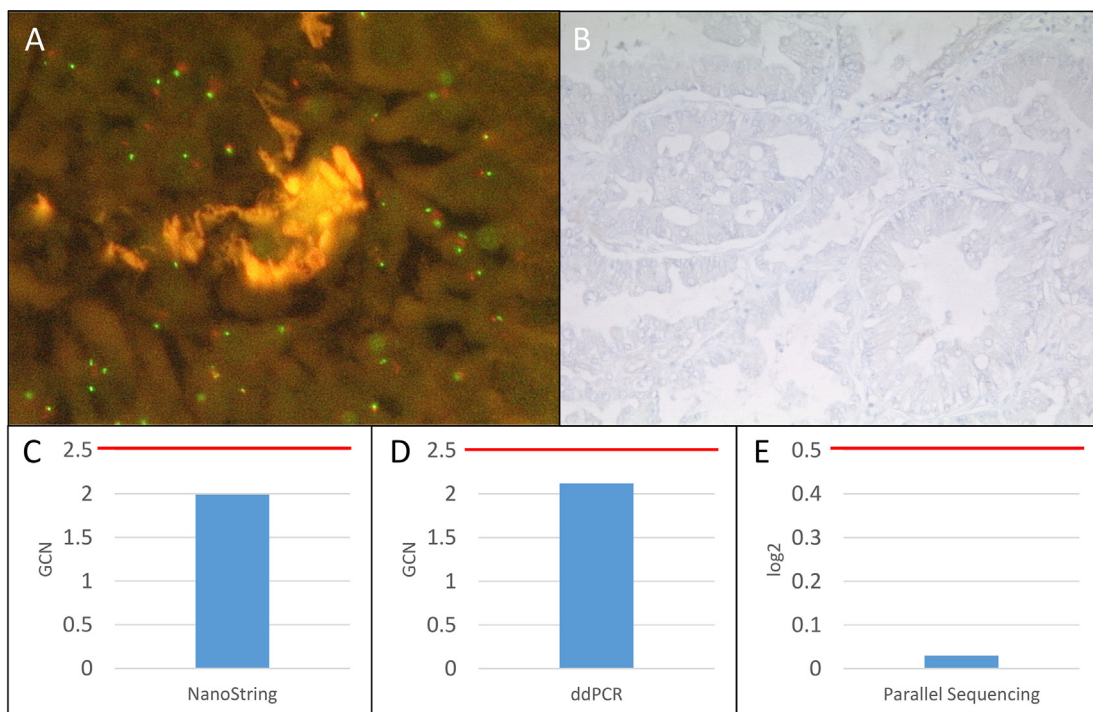
Fig. 1. Summary of *MET* high-level amplified sample 45. A: *MET* FISH high-level (GCN: 29.22) amplification at a magnification of 63x. B: Positive MET IHC (3+) at a magnification of 10x. C: *MET* amplification detected with the NanoString copy number assay (GCN: 25). D: *MET* amplification detected with the ddPCR copy number assay (GCN: 29.2). E: *MET* amplification detected with the custom amplicon-based parallel sequencing assay (log2: 2.55). GCN: Gene copy number. Red bar: Cut-off positivity.
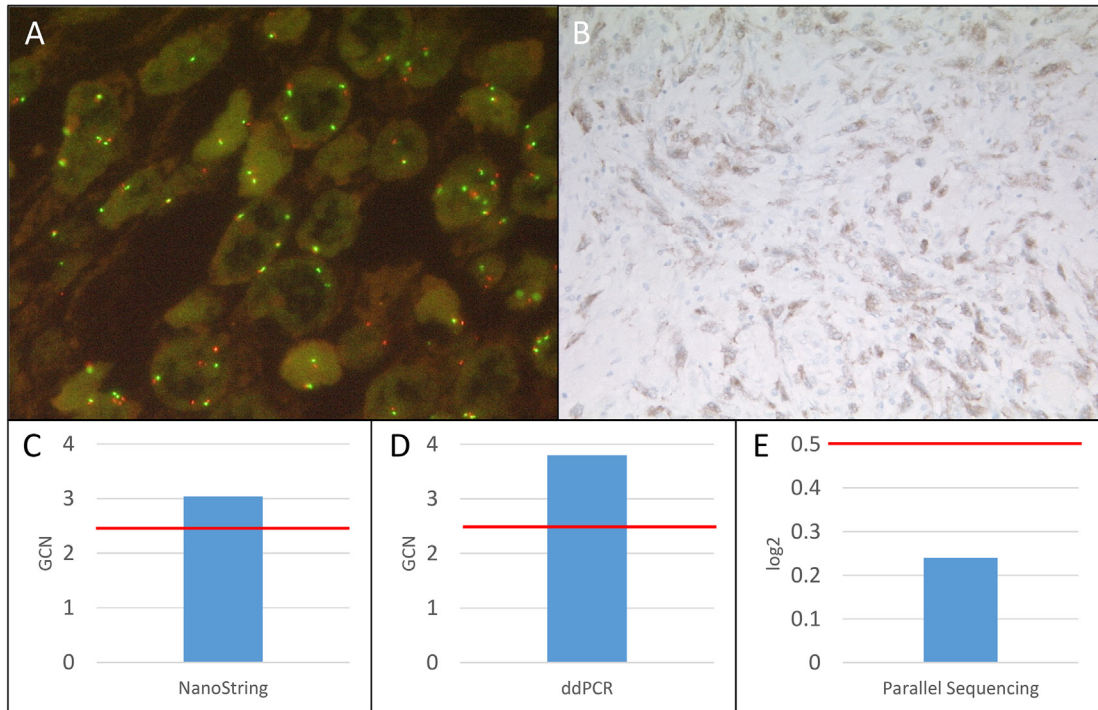

Fig. 2. Summary of *MET* FISH high-level amplified sample 41. A: *MET* FISH high-level (GCN: 6.38) amplification at a magnification of 63x. B: Positive MET IHC (2+) at a magnification of 10x. C: No *MET* amplification detected with the NanoString copy number assay (GCN: 2.24). D: No *MET* amplification detected with the ddPCR copy number assay (GCN: 2.00). E: No *MET* amplification detected with the custom amplicon-based parallel sequencing assay (log2: −0.08). GCN: Gene copy number. Red bar: Cut-off positivity.
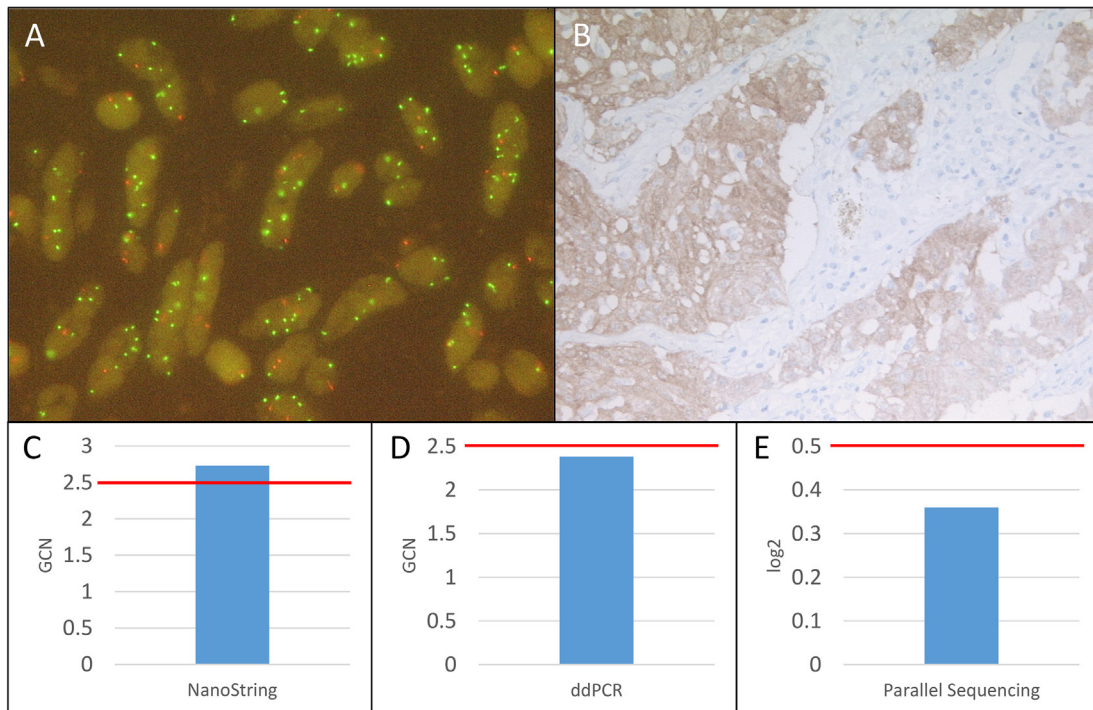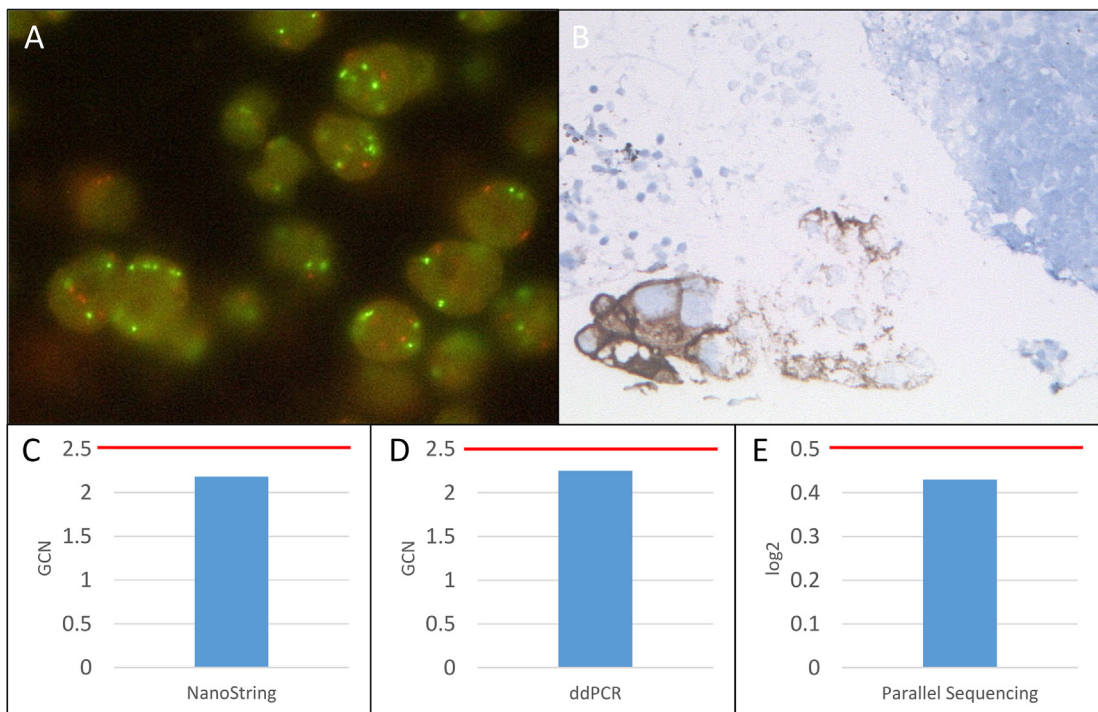
Fig. 3. Summary of *MET* FISH high-level amplified sample 32. A: *MET* FISH high-level (Ratio: 2.77) amplification at a magnification of 63x. B: Positive MET IHC (3+) at a magnification of 10x. C: *MET* amplification detected with the NanoString copy number assay (GCN: 2.73). D: No *MET* amplification detected with the ddPCR copy number assay (GCN: 2.38). E: No *MET* amplification detected with the custom amplicon-based parallel sequencing assay (log2: 0.36). GCN: Gene copy number. Red bar: Cut-off positivity.

Fig. 4. Summary of *MET* FISH not amplified sample 4. A: No *MET* FISH amplification at a magnification of 63x. B: Negative MET IHC (0) at a magnification of 10x. C: No *MET* amplification detected with the NanoString copy number assay (GCN: 1.99). D: No *MET* amplification detected with the ddPCR copy number assay (GCN: 2.12). E: No *MET* amplification detected with the custom amplicon-based parallel sequencing assay (log2: −0.03). GCN: Gene copy number. Red bar: Cut-off positivity.

Fig. 5. Summary of *MET* FISH low-level amplified sample 12. A: *MET* FISH low-level (41.67%≥4 *MET* signals) amplification at a magnification of 63x. B: Positive MET IHC (2+) at a magnification of 10x. C: *MET* amplification detected with the NanoString copy number assay (GCN: 3.04). D: *MET* amplification detected with the ddPCR copy number assay (GCN: 3.80). E: No *MET* amplification detected with the custom amplicon-based parallel sequencing assay (log2: 0.24). GCN: Gene copy number. Red bar: Cut-off positivity.

Fig. 6. Summary of *MET* FISH high-level amplified sample 21. A: *MET* FISH intermediate-level (71.67%≥5 *MET* signals) amplification at a magnification of 63x. B: Positive MET IHC (3+) at a magnification of 10x. C: No *MET* amplification detected with the NanoString copy number assay (GCN: 2.18). D: No *MET* amplification detected with the ddPCR copy number assay (GCN: 2.25). E: No *MET* amplification detected with the custom amplicon-based parallel sequencing assay (log2: 0.43). GCN: Gene copy number. Red bar: Cut-off positivity.

fied as positive. In the high-level amplified cohort with a MET/CEN7 ratio ≥2 5 of 10 samples (50%) were positive with a GCN higher than 2.5 and in the high level amplified cohort with a *MET* GCN ≥6 6 of 10 samples (60%) were positive with a GCN ≥2.5. Sample 43 was negative in the ddPCR copy number assay although the GCN determined by FISH was very high with 22.8. This sample had a tumor cell content of only 30%. In summary, in the *MET* high-level amplified cohorts there were only 11 of 20 samples with matching results comparing *MET* FISH and the ddPCR copy number assay (55%) whereas in the other three cohorts 17 of 25 samples concurred (68%) (Table 2, Figs. 1–6).

3.3. NanoString copy number assay

The NanoString copy number assay was performed for all 45 samples (Table 1). In the cohort without *MET* amplification 9 of 10 samples (90%) had a GCN of less than 2.5 and were classified as negative. Only sample 8 was borderline positive with a GCN of 2.8. In the *MET* low-level amplified cohort 2 of 5 samples (40%) and in the *MET* intermediate-level amplified cohort 5 of 10 samples (50%) were positive with a GCN of ≥2.5. In the high-level amplified cohort with a MET/CEN7 ratio ≥2 7 of 10 samples (70%) were positive with a GCN higher than 2.5 and in the high level amplified cohort with a *MET* GCN ≥6 9 of 10 samples (90%) were positive with a GCN ≥2.5. In the latter cohort only sample 41 was negative with a GCN of 2.2, but this sample was flagged by the NanoString copy number assay as not having enough DNA for the assay and the sample was also negative by the ddPCR copy number assay and the custom amplicon-based parallel sequencing assays. In summary, in the *MET* high-level amplified cohorts 16 of 20 samples (80%) showed consistent results with the *MET* FISH whereas in the other cohorts only 16 of 25 samples had matching results (64%) (Table 2, Figs. 1–6).

3.4. Custom amplicon-based parallel sequencing assays

The results of the custom amplicon-based parallel sequencing assays are depicted in Table 1. For all samples parallel sequencing was performed. In the cohort without *MET* amplification 10 of 10 samples (100%) had a log2 less than 0.5 and were classified as negative. In the *MET* low-level amplified cohort 0 of 5 samples (0%) had a log2 higher than 0.5 and in the *MET* intermediate-level amplified cohort 4 of 10 samples (40%) had a log2 higher than 0.5 and were classified as positive. In the high-level amplified cohort with a MET/CEN7 ratio ≥2 4 of 10 samples (40%) were positive with a log 2 higher than 0.5 and in the high level amplified cohort with a *MET* GCN ≥6 5 of 10 (50%) were positive with a log2 ≥0.5. In summary, the *MET* high-level amplified cohorts showed a concordance of 45% (9 of 20 samples) with the *MET* FISH. The other cohorts had matching results in 14 of 25 samples (56%) (Table 2, Figs. 1–6). Comparing the different panels used, LUN3 had a total concordance of 62.5% (5 of 8 samples), LUN4 of 44.8% (13 of 29 samples) and LUN5 of 62.5% (5 of 8 samples). With LUN4 the most samples were analyzed.

Table 3
Estimation of agreement between FISH and alternative testing methods. Positive percent agreement (PPA), negative percent agreement (NPA), and overall percent agreement (OPA) for each comparison was calculated using the definition of positive and negative results as described.

	IHC	NanoString	ddPCR	Parallel sequencing
OPA	0.84	0.62	0.56	0.51
PPA	0.94	0.91	1.00	1.00
NPA	0.62	0.35	0.33	0.31
Kappa	0.59	0.25	0.25	0.21

In summary, the MET IHC had the best agreement with FISH followed by NanoString copy number assay, ddPCR copy number assay and the custom amplicon-based parallel sequencing assays (Table 2, Table 3). The performance of immunohistochemistry testing as well as the extraction-based methods is also described by calculating positive percent agreement (PPA), negative percent agreement (NPA), and overall percent agreement (OPA) (Table 3). Compared to FISH the number of false positives for all methods is low but especially for the extraction-based methods there is a high number of false negatives.

4. Discussion

In EGFR-treatment naive NSCLC patients, high-level *MET* amplification is detected in approximately 2–3% and is considered as adverse prognostic factor [17]. Only few data on treatment of these patients with MET inhibitors are available [18] and two previous clinical studies where patients had been selected based on MET immunohistochemical expression failed [19,20]. A technical comparison on MET expression analysis by immunohistochemistry also showed a high interlaboratory variability and highlighted the need for harmonization of MET IHC [21]. Nevertheless, in other studies MET IHC positivity was significantly ($P < 0.001$, χ^2 test) associated with *MET* high-level amplification and *MET* high-level amplified samples can be selected by high MET IHC scores, which is in concordance with our study [13,22].

Currently, clinical trials with two different inhibitors, capmatinib and tepotinib, are under way [12,23,24] both defining different inclusion criteria regarding *MET* amplification from proven amplification only to defining an exact *MET* copy number.

This highlights the need for an accurate predictive biomarker test prior to MET directed treatment to evaluate the true prevalence of MET overexpression and amplification. In this study, conventional slide-based approaches FISH and IHC were compared with the results of three extraction-based methods, ddPCR, the NanoString nCounter technology and amplicon-based parallel sequencing for the detection of *MET* amplifications. The use of extraction-based methods is not subjected to interobserver variability because a well defined cut-off value by extensive validation studies can be applied. Further, laborious evaluation by a trained pathologist can be avoided.

Depending on the inclusion criteria of clinical studies different extraction-based technologies can be used instead of *MET* FISH or MET IHC. For clinical studies including only *MET* high-level amplified samples with a high gene copy number the NanoString copy number assay gave the best results in comparison to the *MET* FISH. However, this technology is hampered by the large amount of DNA needed for analysis, which is not available for most lung cancer biopsies.

Also for ddPCR, previous studies showed a very strong correlation of the ddPCR copy number assay and *MET* FISH high-level amplification (MET/CEN7 ratio of ≥2), but this is in contrast to our study where only 50% of samples matched [25].

In the extraction-based technologies, however, intratumoral heterogeneity in the signal pattern of the *MET* FISH can result in a lower *MET* copy numbers and thus in false negative results. Partly, this has been reported previously [13]. It was also shown in gastric-/esophageal adenocarcinoma that *MET* amplification was markedly higher in tumor specimens with a heterogeneous signal distribution [26], which might be problematic for the detection by extraction-based technologies. Further, in our study FFPE material with varying DNA quality was used, therefore after extensive validation, literature research [13,14] and manufactures instruction the cut-offs were set quite high to a gene copy number

≥2.5 and a log2 ≥0.5 to decrease the number of false positives by elevated background noise.

For future analysis, *MET* copy number detection by parallel sequencing is the most promising technology as small variants as well as gene copy number changes of many genes and samples can be detected at the same time. However, in our study parallel sequencing data was the least reliable method. In our study, notably a tumor cell content below 50% led to false negative results, as the detection limit was set relatively high to minimize false positive results. In general, gene copy number detection with the custom amplicon-based parallel sequencing assays is very sensitive to tumor complexity like tumor purity and heterogeneity as previously described [27–29].

Further, an amplicon-based parallel sequencing approach without unique molecular identifier were used and the panel of normals consisted of negative samples and not normal tissue, which might have influenced the analysis. In previous studies was shown, that the detection limit increases with a decreasing tumor cell content. Here, amplification calling on calls of more than one amplicon can increase the sensitivity and lower the detection limit [30]. In our study, no differences between the panels using only one amplicon or five amplicons were seen. However, our sample numbers are too small to draw a final conclusion and more research is needed.

In conclusion, our study showed that currently extraction-based methods cannot replace the MET FISH for the detection of low-level, intermediate-level and high-level MET amplifications, as the number of false negative results is very high. Currently, clinical trials with different MET inhibitors are ongoing but the role for MET amplification as predictive biomarker is still ambiguous [31]. This can at least in part be attributed to the inappropriate definition of MET amplification. The outcome of these clinical trials will show the clinical relevance of the different groups of MET amplification status determined by FISH, as the published MET FISH amplification criteria [10] are only descriptive and are not based on clinical screening programs. Extraction-based methods are only able to replace the MET FISH reliably for the detection of high-level amplified samples with a GCN ≥10.

Declaration of Competing Interest

Acknowledgment

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