A NOVEL RT-ddPCR ASSAY ENABLES SENSITIVE AND ACCURATE QUANTIFICATION OF *MET* EXON 14 SKIPPING IN LUNG ADENOCARCINOMAS

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SUMMARY

- *MET* experiences exon 14 loss (*MET*ex14) in 3-4% of lung adenocarcinomas, thereby promoting protein stabilization and oncogenic pathway activation.
- Exon 14 loss or elevated *MET* levels are clinically actionable events susceptible to kinase inhibitors, including crizotinib and cabozantinib.
- Droplet Digital™ PCR (ddPCR™) can provide accurate copy number information of both wildtype and *MET*ex14 status using a rapid and accessible workflow.
- We describe a prototype multiplexed ddPCR assay that quantifies an internal reference gene and the fraction of *MET*ex14 skipping.

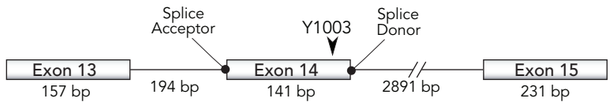
INTRODUCTION

The proto-oncogene *MET* experiences donor/acceptor splice site mutations that can give rise to transcripts lacking exon 14 (*MET*ex14)[1]. Although this "skipping" event occurs at frequencies comparable to *ALK* rearrangements in lung adenocarcinomas, current *MET*ex14 detection technologies are underdeveloped and burdened by a combination of high costs, low resolution, and/or complex workflows. Herein we report the development of a novel, accurate, and accessible 2-channel Droplet Digital PCR (ddPCR) assay that can quantify the fraction of skipped *MET*ex14 transcripts in patient samples. Successful identification of this event correlates with MET inhibitor responsiveness, potentially benefiting treatment decisions and outcomes[2].

MATERIALS AND METHODS

A *MET*ex14 RT-ddPCR assay was developed using optimized reverse transcription and PCR reagents, with an optional pre-amplification step incorporated for samples with low nucleic acid quantity or quality. Gene-specific primers and junction-spanning hydrolysis probes were designed for *MET* exons 13/14 or 14/15 (wildtype (WT), HEX labeled) and 13/15 (*MET*ex14, FAM labeled), respectively. Droplets were generated and run on the QX200™ ddPCR platform (Bio-Rad) with a 4-bin output. Samples consisted of cell lines and a set of 125 lung adenocarcinoma FFPEs (MD Anderson) that were sequenced in parallel for *MET*ex14 using the QuantideX® NGS RNA Lung Cancer Kit* (Asuragen).

1A



1B

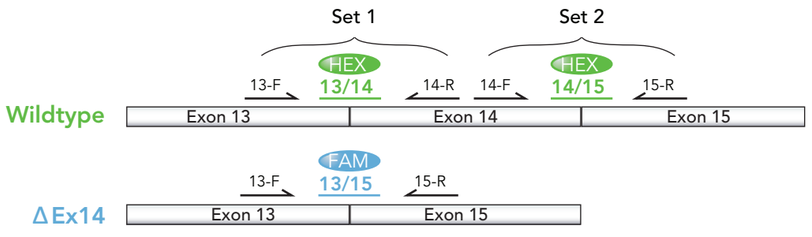


Figure 1. Assay design for detection of *MET*ex14 status. A) Pertinent *MET* genomic intron/exon junctions. Wide-ranging indels and point mutations cluster around donor & acceptor sites, leading to excision of exon 14 during splicing and the subsequent removal of a negative CBL regulation site codon (Y1003). **B)** Assay primer & fluorescent probe placement options for detecting processed transcripts.

*Research Use Only – Not For Use In Diagnostic Procedures
Preliminary research data. The performance characteristics of this assay have not yet been established.

Presented at AMP 2016 - S86

RESULTS

MET exon 14 skipping status was determined with 100% accuracy in representative cell lines from as little as 2 ng RNA inputs. Known copy number titrations demonstrated analytical sensitivity down to 1% *MET*ex14 skipped/WT levels, exceeding the sensitivity typically achieved by other approaches. *MET*ex14 positive and negative samples were correctly identified from previously characterized FFPE specimens, including two samples that showed 27% and 95% *MET*ex14 skipping ratios, respectively.

2A

	Probe	Copies/20uL 13/15	Copies/20uL 13/14		Accepted Droplets
	Sample	Skipped	WT	% Skipped	
Positive cell line	H596- 50ng	80,600	350	99.6%	9,996
	H596- 10ng	12,860	54	99.6%	13,203
	H596- 2ng	2,900	14	99.5%	13,700
Wildtype	RT112-50ng	0	5,180	0.0%	15,465
	RT112-10ng	0	1,068	0.0%	12,777
	RT112-2ng	0	184	0.0%	14,243
Controls	RT-NTC	0	0	-	13,336
	PCR-NTC	0	0	-	11,131

2B

Sample	Copies/20uL 13/15	Copies/20uL 13/14	% Skipped	Avg%
H596-2ng	3,680	0	100.0%	100%
H596-2ng	3,700	1	99.9%	
RT112-30ng	1	2,900	0.1%	0.1%
RT112-30ng	0	3,060	0.0%	
25% Positive	1,040	2,480	29.5%	28.6%
25% Positive	858	2,240	27.7%	
12% Positive	446	2,500	15.1%	15.4%
12% Positive	484	2,600	15.7%	
6% Positive	202	2,680	7.0%	6.9%
6% Positive	226	3,060	6.9%	
1.2% Positive	42	2,900	1.4%	1.6%
1.2% Positive	58	3,140	1.8%	
RT-NTC	0	0	-	-
RT-NTC	0	0	-	

2C

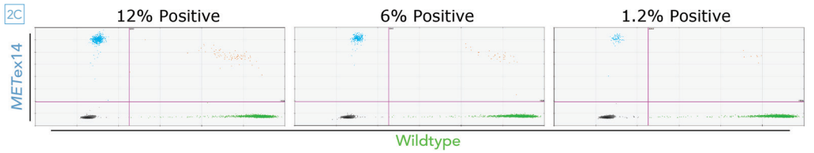
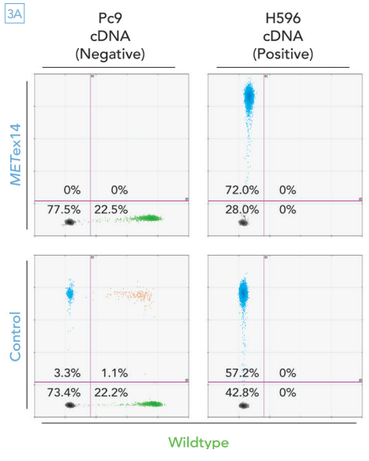


Figure 2. *MET*ex14 ddPCR detects low-frequency skipping events with high specificity. A) Absolute ng input detection for H596 (*MET*ex14 positive) and RT112 (wildtype *MET*) cells. **B)** *MET*ex14 (H596) cDNA was titrated into a wildtype (RT112) background based upon levels established in the originating samples. **C)** Visualization of select data from B.

3A



3B

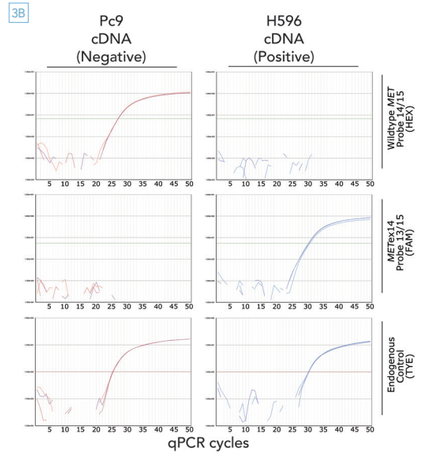
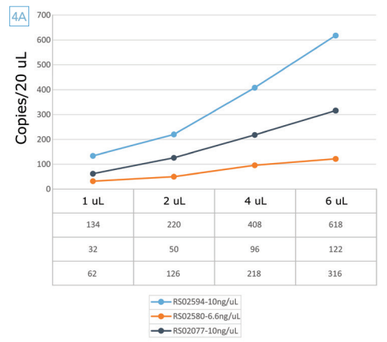


Figure 3. An endogenous reference gene can be incorporated into multiplex *MET*ex14 ddPCR or qPCR assay configurations. A) Inclusion of a stable endogenous reference gene as an internal control. Two separate wells detect the presence of wildtype(14/15)/skipped(13/15) *MET* and wildtype/control transcripts, respectively. Shared *MET* 14/15 quantitation serves as an additional cross-reaction control. **B)** As an alternative to ddPCR, a single-reaction 4-channel qPCR can be performed with ddPCR probe/primer components, including the endogenous control. No off-targeting was noted.

4A



4B

	Sample	ddPCR Input Dilution Factor	cp 13/15	cp 14/15	% Skipped
Standard Workflow	H596	1	6,800	0	100.0
	H596/RT112 (2.5% Pos.)	1	106	6,860	1.5
	RS02181 (FFPE)	1	1	3,200	0.0
	RS02272 (FFPE)	1	1	624	0.2
	RS02241 (FFPE)	1	0	122	0.0
	NTC	1	0	0	–
Pre-Amplification	H596	10	66,600	0	100.0
	H596/RT112 (2.5% Pos.)	10	1,318	58,600	2.2
	RS02181 (FFPE)	10	0	22,000	0.0
	RS02272 (FFPE)	10	0	4,640	0.0
	RS02241 (FFPE)	10	0	774	0.0
	NTC	10	0	0	–
	H596	100	6,560	0	100.0
	H596/RT112 (2.5% Pos.)	100	126	5,880	2.1
	RS02181 (FFPE)	100	0	2,500	0.0
	RS02272 (FFPE)	100	0	480	0.0
	RS02241 (FFPE)	100	0	84	0.0
	NTC	100	0	0	–

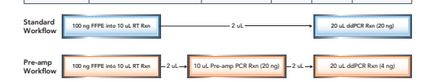


Figure 4. *MET*ex14 ddPCR optimizations increase cDNA inputs into the PCR stage by at least 100-fold. A) Increasing RT loading by as much as 30% of the ddPCR volume was well tolerated. Wildtype *MET* FFPEs were tested from Asuragen's Tumor Bank (14/15 probe). **B)** An optional pre-amplification stage significantly improved sensitivity while preserving allele ratios.

5A

3' Fusion Genes	# of Fusions
ALK	53
ROS1	22
RET	12
FGFR3	7
NTRK3	3
NTRK1	4

3' Fusion Genes	# of Fusions
NRG1	2
FGFR1	1
FGFR2	1
MBIP	1
PDGFRA	1

Exon Assessment
MET e13:e14
MET e14:e15
MET e13:e15

5B

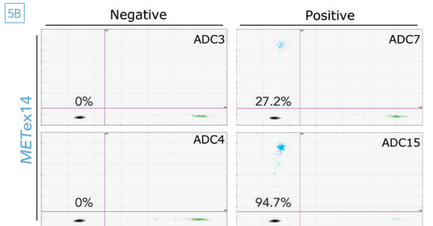


Figure 5. Accurate detection of *MET*ex14 positive and negative samples from residual FFPE lung cancer specimens. A) 125 lung adenocarcinoma FFPE samples (MD Anderson) were screened using the QuantideX RNA Lung Cancer Kit, resulting in the identification of two *MET*ex14 positives. Select NGS panel content, including relevant *MET* transcript isoforms, is shown. *MET*e5:e6 is additionally assessed. **B)** ddPCR data for representative FFPE negatives (samples ADC3 & ADC4) and the two identified positives (ADC7 & ADC15) following pre-amplification and a 1:100 fold input dilution. Percent skipped vs. wildtype *MET* noted.

CONCLUSIONS

- The prevalence and druggability of *MET*ex14 skipping in NSCLC makes this lesion a high-priority diagnostic target for precision medicine.
- We describe a prototype ddPCR assay that can accurately quantify the fraction of *MET*ex14 within a single multiplexed reaction.
- Further inclusion of a stable reference gene enables relative quantification of wildtype *MET* transcript expression independent of mechanism, and can be accommodated by both ddPCR and qPCR formats.
- The assay accepts challenging clinical specimen inputs (e.g. FFPEs) and confirms *MET*ex14 status as identified using the Asuragen QuantideX NGS RNA Lung Cancer Kit (RUO)*.

References

1. Kong-Beltran et al. Somatic mutations lead to an oncogenic deletion of *MET* in lung cancer. Cancer Res. (2006). 66: 283-289.
2. Paik et al. Response to *MET* inhibitors in patients with stage IV lung adenocarcinomas harboring *MET* mutations causing exon 14 skipping. Cancer Discov. (2015). 8: 842-849.

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