National Institute of Standards & Technology

Report of Investigation

Reference Material 8366

EGFR and *MET* Gene Copy Number Standards for Cancer Measurements

Reference Material (RM) 8366 is intended to harmonize the measurements of ratios of the human epidermal growth factor gene (*EGFR*) and human MET proto-oncogene, receptor tyrosine kinase gene (*MET*) to unamplified reference genes. *EGFR* gene amplification and the associated increased protein expression have been reported and implicated in the pathogenesis of many human malignancies. The amplification (increased copies) of the *EGFR* gene and protein overexpression in several types of cancer are used as a biomarker for determining the therapeutic treatment and predicting clinical outcome in response to anti-EGFR targeted therapy [1]. *MET* gene amplification resulting in increased protein expression and constitutive activation of the MET receptor has been described in lung cancer, gastric carcinoma and hepatocellular carcinoma. Various clinical trials were conducted to evaluate the safety and efficacy of selective MET inhibitors in cancer patients. However, the accurate and reliable assessment of MET levels is still a challenge [2]. RM 8366 consists of genomic DNA extracted from six human cancer cell lines with different amounts of amplification of the *EGFR* and *MET* genes. The six purified genomic DNAs are in a buffer consisting of 10 mmol/L 2-Amino-2-(hydroxymethyl) propane-1,3-diol (Tris) and 0.1 mmol/L ethylenediaminetetraacetic acid disodium salt (EDTA) pH 8.0 (TE^{-4}). The six components are genomic DNA materials derived from human cell lines A-431, BT-20, C32, Daoy, Hs 746T, and SNU-5, and labeled A, B, C, D, E, and F, respectively. The approximate concentration of components A, B, C, D, E, and F determined by absorbance measurements at 260 nm are 21.6 µg/mL, 21.7 µg/mL, 21.5 µg/mL, 22.9 µg/mL, 23.1 µg/mL, and 21.0 µg/mL, respectively. It is important to consider that the levels of *EGFR* and *MET* amplification in the individual components when preparing the dilutions to ensure that the *EGFR* and *MET* copy number is within the working range of the assay that you are using. A unit of the RM consists of one vial of each component, containing approximately 100 µL of DNA solution. Each of these vials is labeled and is sealed with a color-coded screw cap.

Reference Values: Reference ratios of *EGFR* and *MET* copies per reference genes with the 95 % credible intervals and 95 % prediction intervals are shown in Tables 1 and 2. A NIST reference value is a best estimate of the true value provided on a NIST certificate, certificate of analysis, or report of investigation where all known or suspected sources of bias have not been fully investigated by NIST [3].

Information Values: Information values for *EGFR, MET,* and Reference Genes Copies per microliter are shown in Table 3. An information value is considered to be a value that will be of interest and use to the RM user, but insufficient information is available to assess the uncertainty associated with the value or only a limited number of analyses were performed [3]. Information values cannot be used to establish metrological traceability.

Expiration of Value Assignment: The reference and informational values of **RM 8366** are valid within the measurement uncertainty specified, until **31 December 2022**, provided the RM is handled and stored in accordance with the instructions given in this report (see "Instructions for Handling, Storage, and Use"). This material and associated information are nullified if the RM is damaged, contaminated, or otherwise modified.

Maintenance of RM: NIST will monitor this RM over the period of its validity. If substantive technical changes occur that affect the value assignment before the expiration of this report, NIST will notify the purchaser. Registration (see attached sheet or register online) will facilitate notification.

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Overall direction and coordination of the technical measurements leading to the report of RM 8366 were provided by K.D. Cole and H.-J. He of the NIST Biosystems and Biomaterials Division.

Sample preparation, analytical measurements, and analyses were done by H.-J. He; K.D. Cole; J. Almeida of the NIST Biosystems and Biomaterials Division. Additional measurements were supplied by M. Cleveland and C.R. Steffen of the Applied Genetics Group in the Biomolecular Measurements Division.

Statistical consultation was provided by Steven Lund of the NIST Statistical Engineering Division.

Support aspects involved in the issuance of this RM was coordinated through the NIST Office of Reference Materials.

NOTICE AND WARNING TO USERS

RM 8366 IS A HUMAN-SOURCE MATERIAL. SINCE THERE IS NO CONSENSUS ON THE INFECTIOUS STATUS OF EXTRACTED DNA, HANDLE THE RM 8366 COMPONENTS AS BIOSAFETY LEVEL 1 MATERIALS CAPABLE OF TRANSMITTING INFECTIOUS DISEASE [4]. RM 8366 components and derived solutions should be disposed of in accordance with local, state, and federal regulations.

INSTRUCTIONS FOR STORAGE AND USE

Storage: All vials of RM 8366 should be stored in the dark between 2 °C to 8 °C. **DO NOT FREEZE.**

Use: Component vials should be mixed briefly and centrifuged (without opening the vial cap) prior to removing sample aliquots for analysis. For the reference and informational values to be applicable, materials should be withdrawn immediately after opening the vials and processed without delay. Dilutions of these materials may be made as appropriate, but the dilutions should be used immediately and not stored. Reference and informational values do not apply to any material remaining in recapped vials. There is no minimum sample size requirement, the amount required by any given assay will be dictated by the nature of the assay. **DO NOT EXPOSE ANY DNA SOLUTION TO DIRECT SUNLIGHT**. This reference material is suitable only for measurement techniques that utilize purified DNA.

For use with digital PCR polymerase chain reaction (dPCR) Assays: The provided reference ratios could be used for *EGFR* and *MET* ddPCR assay validation, optimization, and process evaluation, and measurements harmonization. Use the informational value of *EGFR* and *MET* and reference genes copies per microliter and the approximate concentration values when designing dilutions.

Reference Values: Tables 1 and 2 have reference values for the ratios of the *EGFR* and *MET* gene copies to the average copies of the indicated reference genes, the approximate 95 % credible intervals, and the approximate 95 % prediction intervals. The measurand is the ratio of *EGFR* or *MET* copies to the average copy number of the listed reference genes. Metrological traceability is to the natural unit ratio one [5].

The gene abundance ratio, $Ratio_s$, is defined as:

$$Ratio_s = {Target_s}\Big/{\frac{1}{Gs}\sum_{g=1}^{Gs} Ref_{sg}},$$

where s denotes one of the six components included in RM 8366, Gs denotes the number of reference genes considered for component s, g denotes one of the reference genes, $Target_s$ denotes the measured abundance of *EGFR* or *MET* gene in sample s, and Ref_{sg} denotes the measured abundance of reference gene g in sample s.

The values in Tables 1 and 2 were calculated by fitting a statistical model to the measurements made on the RM 8366 materials using a digital PCR method. The Bayesian paradigm with vague priors was used for statistical inference [6]. The posterior credible interval is an interval calculated in a manner consistent with the ISO/JCGM Guide [7]. The nominal coverage for the interval is 95 % and can be interpreted in the following manner. The NIST analysts believe that the ratio of gene copy number for either *EGFR* or *MET* and the average among the selected set of reference genes (as listed in the "Reference Genes Used for Analysis" column of Table 1 and Table 2) for a single bottle sampled from among those prepared for this RM lies within the respective provided interval with approximately 95 % probability. The prediction interval provides the approximate range of values NIST would reasonably expect upon the next independent, triplicate measurement of the *EGFR* or *MET* copy number ratios to the average among the selected set of reference genes (as listed in the "Reference Genes Used for Analysis" column of Table 1 and Table 2) for each of the six components, based upon the measurement performance of NIST analysts and instruments. The value would be expected to fall within the interval approximately 95 % of the time.

Table 1. Reference Values of the Ratios of *EGFR* Copies to Reference Gene Copies

Component	Cell Line	Color Code	*EGFR* Ratio	Credible Interval 95 %	Prediction Interval 95 %	Reference Genes Used for Analysis
A	A-431	white	6.4	6.2 – 6.7	6.0– 6.8	*EIF5B, RPS27A, DCK, PMM1*
B	BT-20	clear	5.5	5.2 – 5.7	5.1 – 5.9	*EIF5B, RPS27A, DCK*
C	C32	yellow	0.8	0.8– 0.8	0.7 – 0.8	*EIF5B, RPS27A, DCK, PMM1*
D	Daoy	blue	2.2	2.1 – 2.3	2.0 – 2.3	*EIF5B, RPS27A, PMM1*
E	Hs 746T	red	1.3	1.3 – 1.4	1.2 – 1.4	*RPS27A, DCK, PMM1*
F	SNU-5	green	1.8	1.8– 1.9	1.7– 2.0	*EIF5B, RPS27A, DCK*

Table 2. Reference Values of the Ratios of *MET* Copies to the Reference Gene Copies

PREPARATION AND ANALYSIS [1]

Component	Cell Line	Color Code	*MET* Ratio	Credible Interval 95 %	Prediction Interval 95 %	Reference Genes Used for Analysis
A	A-431	white	0.9	0.9– 0.9	0.8– 1.0	*EIF5B, RPS27A, DCK, PMM1*
B	BT-20	clear	1.1	1.1 – 1.2	1.0 – 1.2	*EIF5B, RPS27A, DCK*
C	C32	yellow	2.9	2.8– 3.0	2.7 – 3.1	*EIF5B, RPS27A, DCK, PMM1*
D	Daoy	blue	2.2	2.1 – 2.3	2.1 – 2.4	*EIF5B, RPS27A, PMM1*
E	Hs 746T	red	16.7	16.0 – 17.4	15.6 – 17.8	*RPS27A, DCK, PMM1*
F	SNU-5	green	7.7	7.4– 8.1	7.2– 8.2	*EIF5B, RPS27A, DCK*

Homogeneity and Stability Studies: Each of the components of RM 8366 was distributed into 550 tubes that were then stored at 4 °C in the dark. Homogeneity studies were accomplished by selecting 10 vials of each of the components that were distributed throughout the order of dispensing. These vials were analyzed by a dPCR method. Analysis of the data did not indicate any detectable changes in the values of any of the 6 components that varied with dispensing order (given the uncertainty of the measurements). Stability testing was conducted on the samples that were stored at 4 °C in the dark for different periods of time. The *EGFR* and *MET* copy number, and the ratio of *EGFR* and *MET* gene to the reference gene *RPS27A* and *EIF5B* were measured by dPCR and the data did not show any significant changes in any of the 6 components (given the uncertainty of the measurements) for periods of time up to 408 days, the last storage time analyzed.

Sample Preparation: NIST RM 8366 consists of genomic DNA samples prepared from six human cancer cell lines. After the initial extraction using the Zymo Quick-gDNA™ midiPrep kit (Zymo Research Corporation, Irvine, CA); the samples were pre-treated with bovine pancreatic ribonuclease A before re-extraction [8]. All purified genomic DNA samples were dissolved or eluted in TE^{-4} buffer (10 mmol/L Tris, 0.1 mmol/L EDTA, pH 8.0) and stored at 4 ºC. The DNA samples from the cell lines were genotyped using STR analysis and the results agree with the values provided by the cell line repository.

dPCR Assays: The qPCR assays for *EGFR* and *MET* and reference genes were developed according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [9]. The primers and probes for the *EGFR* and *MET* and reference genes for the qPCR assays are listed in Table 4. The efficiency and specificity of the assays were also determined [10]. MIQE guidelines for dPCR were followed and included sample replicates, positive and negative controls, and proper documentation [11]. TaqMan probes are shown in Table 5. BHQ-1™ (Black hole quencher) and FAM labeled probes were obtained from Biosearch Technologies (Novato, CA). The TaqMan PCR reaction mixture consists of 1 × Droplet dPCR Supermix for probes (no dUTP) (Bio-Rad, Hercules, CA), 900 nmol/L primers, and 250 nmol/L probe (final concentrations) and genomic DNA template or non-template control in a total volume of 25 µL. Approximately 20 ng of genomic DNA was applied in each reaction except for *EGFR* detection in RM 8366 Comp A and B, and *MET* detection in Comp E and F, where 4 ng of genomic DNA was applied. Twenty microliters out of the 25 µL droplet dPCR reaction mixture was used for droplet generation and the

[1] Certain commercial equipment, instruments or materials are identified in this report to adequately specify the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

PCR reaction. The PCR was performed on an Applied Biosystems ProFlex™ PCR System. The following thermal cycling conditions were used: 95 °C for 10 min, followed by 40 cycles of 94 °C for 30 s and 60 °C for 1 min, then 98 °C for 10 min, temperature ramp rate at 2 °C/s. After PCR, the 96-well PCR plate was loaded onto the QX200 droplet digital reader (Bio-Rad), which automatically reads the droplets from each well of the plate. The concentrations of DNA copies were calculated using Poisson sampling statistics assuming that the DNA molecules are partitioning independently from each other into the individual droplets. The results were obtained from assays performed by two analysts using a Bio-Rad QX200 droplet digital PCR system; the Bio-Rad QuantaSoft Version 1.7.4.0917 used a droplet size of 0.85 nL for determination of DNA copy concentrations.

Informational Values: The approximate concentration of components A, B, C, D, E, and F determined by absorbance measurements at 260 nm are 21.6 µg/mL, 21.7 µg/mL, 21.5 µg/mL, 22.9 µg/mL, 23.1 µg/mL, and 21.0 µg/mL, respectively. The absorbance at 260 nm of the components (six replicates) were measured and a conversion factor (one optical density at 260 nm with a pathlength of 1 cm is 50 µg DNA/mL) was used for the calculation. The approximate values were used to determine the appropriate dilution of the samples for use with the desired measurement technique. It is important to consider the level of *EGFR* and *MET* amplification in the samples and appropriate dilutions may be needed to ensure that the *EGFR* and *MET* copy number are within the working range of the assay.

Tables 3 contains the informational values for the concentrations (copies per microliters) of the *EGFR* and *MET* gene and the reference genes in the components of RM 8366, determined by dPCR. This data is from a subset of the data used to calculate the reference ratios in Tables 1 and 2. The samples were measured by dPCR using the reference genes indicated in the respective tables.

Table 3. Informational Values for *EGFR*, *MET* and Reference Genes Copies per Microliter by dPCR[(a)]

Component	*EGFR*	*MET*	Reference Genes used for Measurements	Mean of Reference Genes
A	36691	5132	*EIF5B, RPS27A, DCK, PMM1*	5717
B	34485	6956	*EIF5B, RPS27A, DCK*	6289
C	5279	19618	*EIF5B, RPS27A, DCK, PMM1*	6743
D	15441	15838	*EIF5B, RPS27A, PMM1*	7167
E	7250	90147	*RPS27A, DCK, PMM1*	5402
F	11559	48456	*EIF5B, RPS27A, DCK*	6284

[(a)] Mean values of N=10 samples, the average coefficient of variation for the measurements was 9.4 %.

Table 4. PCR Primer Information for *EGFR*, *MET* and Reference Genes

Primer Name[(a)]	Sequence	PCR Amplicon	Gene Name	Location (GRCh37/hq19)
EGFR-2F	ACCTTTGCAGAGAGGCTTAAT	112 bp	*EGFR*	Intron 1 (chr7:55177325-55177346)
EGFR-2R	CCTAGGCCCAAAGGAATGATAG			(chr7:55177415-55177437)
MET-2F	TGGGCATGCTCATTCTTCTT	91 bp	*MET*	Intron 2 (chr 7:116365077-116365097)
MET-2R	CATCATACTTCTTACGTACAGGCA			(chr 7:116365144-116365168)
EIF5-F	GGCCGATAAATTTTTGGAAATG	112 bp	*EIF5B*	Intron 1 (chr2:99974140-99974161)
EIF5-R	GGAGTATCCCCAAAGGCATCT			(chr2:99974231-99974251)
2PR4-F	CGGGTTTGGGTTCAGGTCTT	97 bp	*RPS27A*	Intron 4 (chr2:55462316-55462335)
2PR4-R	TGCTACAATGAAAACATTCAGAAGTCT			(chr2:55462386-55462412)
R4Q5-F	CTCAGAAAAATGGTGGGAATGTT	122 bp	*DCK*	Exon 3 (chr4:71888097-71888119)
R4Q5-R	GCCATTCAGAGAGGCAAGCT			(chr4:71888199-71888218)
22C3-F	AGGTCTGGTGGCTTCTCCAAT	78 bp	*PMM1*	Intron 7 (chr22:41973739-41973759)
22C3-R	CCCCTAAGAGGTCTGTTGTGTTG			(chr22:41973682-41973704)

[(a)] F: Forward primer
R: Reverse primer

Table 5. TaqMan® Fluorescent Probe Sequences

Probe Name	Sequence	5' Label	3' Quencher
EGFR-2P	TGCTCTTAAAGGGATATCCTCTCCTGGT	FAM	BHQ-1
MET-2P	CCTAGAGTGTGGGTTGGCCTTCCTA	FAM	BHQ-1
EIF5-P	TTCAGCCTTCTCTTCTCATGCAGTTGTCAG	FAM	BHQ-1
2PR4-P	TTTGTCTACCACTTGCAAAGCTGGCCTTT	FAM	BHQ-1
R4Q5-P	CCTTCCAAACATATGCCTGTCTCAGTCGA	FAM	BHQ-1
22C3-P	CAAATCACCTGAGGTCAAGGCCAGAACA	FAM	BHQ-1

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