약학박사학위논문

DNA의 특질 분석을 이용한 드롭렛 디지털 PCR 기반의 *EGFR* 돌연변이 검출 방법 연구

Droplet digital PCR-based *EGFR* mutation detection with an internal quality control index to determine the quality of DNA

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Abstract

Droplet digital PCR-based *EGFR* mutation detection with an internal quality control index to determine the quality of DNA

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Formalin-fixed paraffin-embedded tissue (FFPET) samples are invaluable sources for both translational clinical research and molecular *in vitro* diagnostics. However, accurate detection of genetic mutations in FFPET is a major challenge due to artifactual results, due to sample age and quality. In a pre-clinical study, we used 315 non-small cell lung cancer (NSCLC) FFPET-derived DNA (FFPET-DNA) samples to establish sample criteria reflecting the minimum DNA quality suitable for PCR by comparing the results of droplet digital PCR-based mutation test (ddEGFR test) and qPCR-based EGFR mutation test (cobas® EGFR test). Using this criteria, we conducted a retrospective comparative clinical study of the ddEGFR and cobas EGFR tests of 171 NSCLC FFPET-DNA samples. Based on the pre-clinical study, the FFPET-DNA sample

criterion was established as internal quality control (iQC) index ≥ 0.5 (iQC copies ≥ 500, using 3.3 ng [1000 genome equivalents] of FFPET-DNA), indicating that more than half of the input DNA was amplifiable. Based on this iQC index, an independent clinical study revealed that both tests were significantly concordant (overall percent agreement (OPA) = 92.98%). Discordants not detected by the cobas EGFR test were detected using the ddEGFR test, indicating that the higher sensitivity of the ddEGFR test is due to its lower limit of detection.

iQC index is a reliable indicator of the quality of FFPET-DNA and could be used to prevent incorrect diagnoses arising from low-quality samples. Compared with the cobas EGFR test, the ddEGFR test exhibited superior analytical performance and at least equivalent clinical performance. ddEGFR test is expected to serve as an appropriate treatment guidance for NSCLC patients.

Running title: ddPCR-based *EGFR* mutation detection with DNA iQC index

Key words: *EGFR*, epidermal growth factor receptor; FFPE, Formalin-fixed paraffin embedded; DIN, DNA integrity number; ddPCR, Droplet digital PCR; Genetic mutation detection; iQC, internal quality control, Liquid biopsy; ctDNA, Circulating tumor DNA

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List of Abbreviations

Abbreviation	Word
EGFR	Epidermal growth factor receptor
NSCLC	Non-small cell lung cancer
CDx	Companion diagnostics
TKI	Tyrosine kinase inhibitor
FFPE	Formalin-fixed paraffin embedded
DIN	DNA integrity number
PCR	Polymerase chain reaction
ddPCR	Droplet digital PCR
iQC	Internal quality control
PFS	Progression free survival
SNV	Single nucleotide variation
UDG	Uracil-DNA glycosylase
MPS	Massive parallel sequencing
C/N ratio	Cancer/normal ratio
TPS	Tissue preparation system
gDNA	genomic DNA
PPA	Positive percent agreement
NPA	Negative percent agreement
OPA	Overall percent agreement
PPV	Positive percent value
NPV	Negative percent value

MI	Mutation index
LoB	Limit of blank
LoD	Limit of detection
LoQ	Limit of quantitation
SD	Standard deviation
G.E.	Genomic equivalence
MD	Mutation detected
MND	Mutation not detected
COSMIC	Catalogue of somatic mutations in cancer
CLSI	Clinical and laboratory standards institute
MFDS	Ministry of food and drug safety
cfDNA	Cell free DNA
ctDNA	Circulating tumor DNA

I. Introduction

1. *EGFR* mutations in NSCLC

In light of recent advancements in personalized medicine, nucleic acid-based diagnostics will play a pivotal role in implementation of targeted therapies. For example, the activation of epidermal growth factor receptor (*EGFR*) pathways results in the initiation of cancer proliferation, metastasis, and angiogenesis. *EGFR* and its relatives are known to be oncogenic drivers in cancers such as lung cancer (1), breast cancer (2), and glioblastoma (3-5).

Lung cancer, which accounts for one-third of cancer-related deaths worldwide, is divided into small cell lung cancer (SCLC; which accounts for 20% of cases) and non-small cell lung cancer (NSCLC; which accounts for the other 80%). SCLC is a neural crest tumor that responds well to chemotherapy, at least initially; however, it often recurs as treatment-resistant disease. NSCLC, thought to be derived from lung epithelial cells, includes various subtypes: adenocarcinoma, bronchioloalveolar carcinoma, squamous cell carcinoma, and anaplastic and large cell carcinoma. Most patients with advanced NSCLC have a very high probability of metastasis and, if not treated, the mean survival time and 1 year survival rate is at 4–5 months less than 10% respectively (6). Combination cytotoxic chemotherapy increases the survival rate only slightly and causes serious non-specific toxicity to the patient (7). However, the problem of chemotherapy has been raised in the appearance of molecular targeted therapeutic drugs at NSCLC, and small molecule inhibitor of *EGFR* kinase for treatment of NSCLC was approved in 2005 (8).

Inhibiting EGFR kinase activity using tyrosine kinase inhibitors (TKIs) such as erlotinib and gefitinib slows disease progression in patients with NSCLC (9-11). *EGFR* kinase domain mutations occur in four exons encoding part of the tyrosine kinase domain of the enzyme and are clustered around the ATP-binding pocket (12). Deletions in *EGFR* exon 19 and the point

mutation L858R constitute about 90% of all *EGFR*-activating mutations in NSCLC. These mutations correlate with a better TKI treatment response rate and with significant increases in progression free survival (PFS) (13-18) (Fig. 1 and Table. 1). Some reports suggest that *EGFR*-activating mutations may be a prognostic factor for EGFR-TKI efficacy.

Despite EGFR-TKIs such as erlotinib or gefitinib being used as a standard treatment for NSCLC patients harboring EGFR mutations (19), the median PFS still does not exceed 10 months (20). This is because of the secondary point mutation T790M, which is associated with acquired resistance to EGFR-TKIs and is found in up to 50% of post-TKI tumor samples (21-24). T790M increases receptor binding affinity for ATP, thereby reducing drug activity. Recent evidence suggests that the T790M mutation is present in tumor cells at low frequency during development of NSCLC, but there is evidence that tumor cells harboring this mutation might be enriched during treatment with EGFR-TKIs (25, 26) (Fig. 2). Also, pre-existing *EGFR* T790M mutations are associated with poor clinical outcome after EGFR-TKI therapy for NSCLC (27, 28).

Therefore, new EGFR-TKIs that bind specifically to T790M-mutated receptors have been developed and used to treat patients with acquired resistance (29-31). Osimertinib (AZD9291), rociletinib (CO-1686), olmutinib (HM61713), and others (Nazartinib, Naquotinib) are undergoing clinical trials, and osimertinib (TAGRISSO™) has been approved by the U.S, FDA as a treatment for T790M-positive NSCLC (32) (Table.2). Although exciting data and response rates have been registered in patients treated with third generation EGFR-TKIs, acquired resistance still occurs after about 10 months (29, 30). C797S is another acquired *EGFR* mutation that inhibits the activity of EGFR-TKIs. The first evidence of C797S isolated from NSCLC patients was reported by Thress *et al* (33). The authors analyzed plasma samples from 19 patients with acquired resistance to osimertinib and identified C797S in six. The C797S

mutation appears to be an ideal target for overcoming acquired resistance to third generation inhibitors. Thus, accurate detection of *EGFR* mutations is critical for individualized treatment strategies for NSCLC.

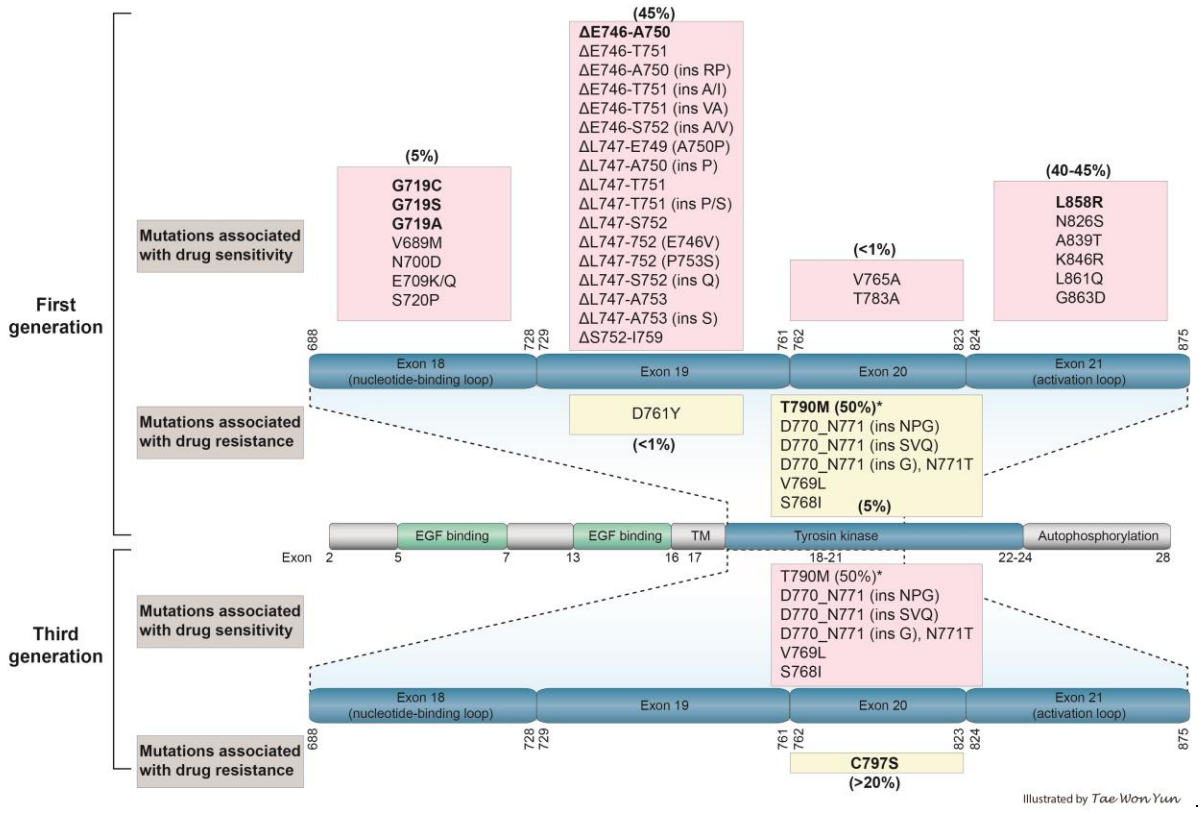

Figure 1. EGFR-TKI sensitizing mutations of *EGFR* in NSCLC. Exons 18–21 in the tyrosine kinase region, in which the relevant mutations are located, are expanded (cyan bars). A detailed list of *EGFR* mutations associated with sensitivity (magenta boxes) or resistance (yellow boxes) to first and third generation TKIs is show for each exon.

Drug	Clinical trial	RR (%)		PFS (months)		OS (months)		Reference
		EGFR-TKI	Platinum-doublet	EGFR-TKI	Platinum-doublet	EGFR-TKI	Platinum-doublet	
Gefitinib	IPASS	71.2	47.3	9.5	6.3	21.6	21.9	(34, 35)
	NEJ002	73.7	30.7	10.8	5.4	30.5	23.6	(17)
	WJTOG3405	62.1	32.2	9.2	6.3	30.9	not reached	(36)
Erlotinib	OPTIMAL	83	36	13.1	4.6	22.6	28.8	(37)
	EURTAC	58	15	9.7	5.2	19.3	19.5	(20)
Afatinib	LUX-Lung 3	56	23	11.1	6.9	not reported	not reported	(38)
	LUX-Lung 6	66.9	23	11	5.6	22.1	22.2	(39)

Table 1. Gefitinib, erlotinib, and afatinib versus platinum-doublet chemotherapy as first line therapy for advanced *EGFR*- mutated NSCLC.

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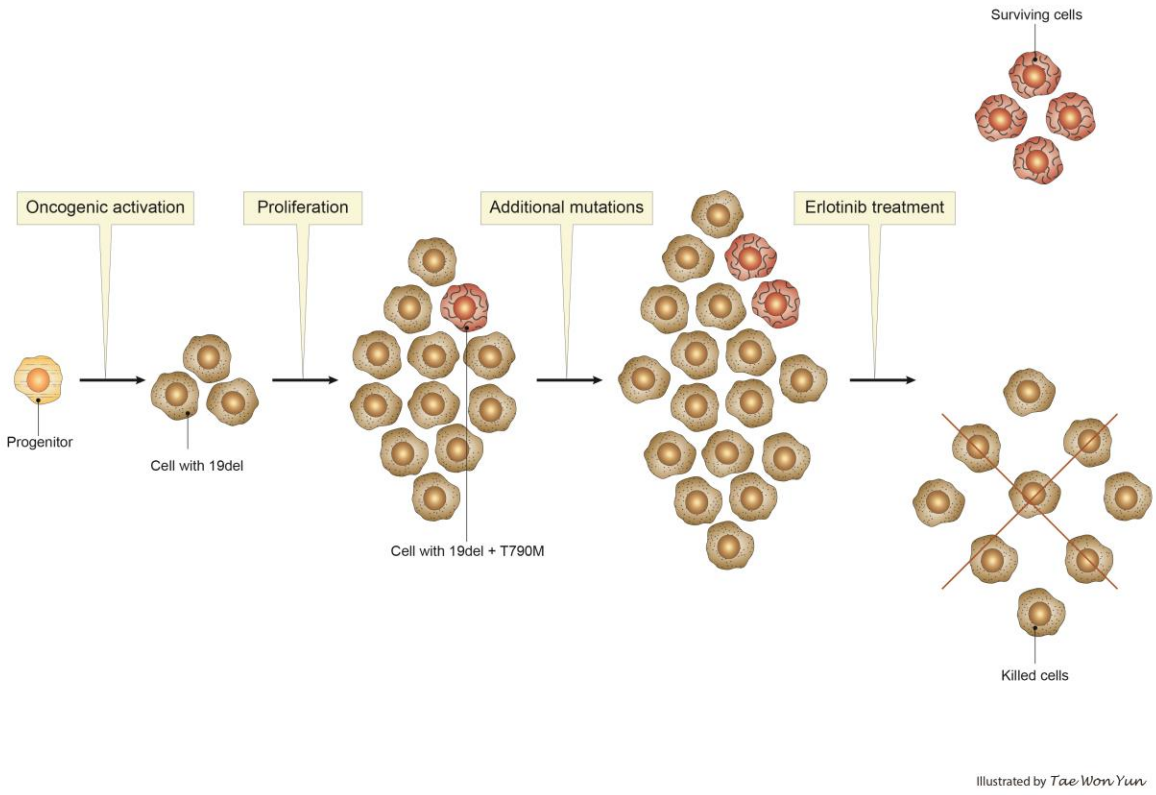


Figure 2. Hypothesis explaining T790M mutation-related resistance to EGFR-TKIs (gefitinib or erlotinib). Treatment with EGFR-TKIs may result in selection of T790M mutant cells. Thus, even if a small number of T790M-positive tumor cells are present at the beginning of treatment, selective proliferation of these cells could lead to clinical resistance to erlotinib.

Drug [research code]	Trial or ClinicalTrials.gov no.*	ORR (%)		PFS (months)		OS (months)		Status	Ref.
		T790M+	T790M-	T790M+	T790M-	T790M+	T790M-		
Osimertinib [AZD9291]	AURA 3	71	31	10.1	4.4	not reported	not reported	Approved	(40)
Rociletinib [CO-1686]	NCT01526928	59	29	13.1	5.6	not reported	not reported	Stopped	(30)
Olmutinib † [HM61713]	ELUXA 1	58.8	-	-	-	-	-	Phase II ongoing	(41)
Nazartinib [EGF816]	NCT02108964	54.5	-	-	-	-	-	Phase II ongoing	(42)
Naquotinib [ASP8273]	NTC02192697	64	-	-	-	-	-	Phase II ongoing	(43)

* *https://clinicaltrials.gov/ct2/home*

† Olmutinib has been approved by the Korea Ministry of Food and Drug Safety (MFDS).

Table 2. Third-generation EGFR-TKIs developed for treatment of NSCLC.

2. Companion diagnostics (CDx)

It is increasingly common for clinicians to analyze molecular characteristics of tumors prior to choosing the most appropriate treatment. When used alongside clinically validated and accurate companion diagnostics, the therapeutic effect of EGFR-TKI can be maximized.

In July 2011, the U.S. FDA announced guidelines on the use of companion diagnostics as an important tool for guiding the selection and use of appropriate therapies (*In vitro* companion diagnostics devices – guidance for industry and FDA staff, US FDA, 2014). According to this definition, CDx analysis is an *in vitro* diagnostic device that provides essential information for the safe and effective use of therapeutic drugs. The FDA also specifies three areas in which a CDx assay is required: [i] to identify patients most likely to benefit from a specific therapeutic product; [b] to identify patients who may suffer serious side effects as a result of treatment with a specific therapeutic product; [c] to monitor treatment responses and adjust the regimen (e.g., drug dosage, treatment time) to increase safety and efficacy. The aim of companion diagnostics is to thoroughly understand the molecular pathology and mechanism of action of a drug so that the properties of a particular molecule are suited to the required treatment outcome.

A previous report highlights the importance of companion diagnostics comprising trastuzumab (Herceptin®, Roche/Genentech) and immunohistochemical (IHC) analysis of HER2-positive advanced breast cancer (44). In 1998, the FDA approved trastuzumab and IHC for analysis of HER2 overexpression (HercepTest™, Dako); since then, several new targeted anticancer agents have been approved and introduced into the clinic. If an appropriate drug and dose are to be tailored to suit a particular individual, robust and accurate analysis methods that have a short turnaround time are needed. Important factors include standardization of analytical methods, validation of reagents and methods, qualified laboratory experience, and involvement

of pathologists. However, recent quality assurance studies conducted to confirm the mutation status of a standard panel of tumors indicate that, despite using the same or similar methods, laboratories may obtain different results (45, 46). Not only differences in methods, but also differences in the characteristics of the sample tested and environmental factors at each test site, can affect the result; therefore, it is imperative that all procedures are standardized.

Many techniques were developed instead of direct sequencing for *EGFR* mutation analysis. This is because direct sequencing has low sensitivity, is complex and time consuming, and is very difficult to standardize (47). Therefore, new techniques have been developed to detect known and *de novo* mutations, albeit with varying sensitivity (48). The Roche Cobas® EGFR Mutation Test (Roche diagnostics, Basel, Switzerland) detects 42 mutations in *EGFR* exons 18, 19, 20, and 21. In parallel with approval of osimertinib as a second-line treatment for patients harboring the T790M mutation, the Cobas EGFR test has been approved by the U.S. FDA. In particular, the assay overcomes the limitation of requiring tumor tissues because *EGFR* mutation status is assessed in a non-invasive manner. The safety and effectiveness of the Cobas EGFR test was confirmed by retrospective clinical validation studies using specimens from NSCLC patients screened in the EURTAC trial (49) and establishment through AURA 3 clinical trial using matched plasma to the tissues (40). This test allows for *EGFR* mutation screening in a single reaction with a 1-day turnaround time, and has greater sensitivity and specificity than Sanger sequencing.

In July 2013, the Scorpion Amplified Refractory Mutation System (ARMS) therascreen® EGFR Rotor-Gene® Q PCR kit (therascreen EGFR test; QIAGEN, Hilden, Germany) was approved by the U.S. FDA for use as a companion diagnostic for afatinib. A recent study reported a high concordance rate between the therascreen EGFR test and the Cobas EGFR test (50). However, the Cobas EGFR test has some advantages. For example, the time from

obtaining a tumor sample to obtaining a result is shorter, meaning that treatment can begin more quickly. In addition, Cobas EGFR test requires a very small amount of DNA to detect the mutation status of a tumor sample. The comparison of the two kits are shown in Table 3.

	Roche Cobas® EGFR mutation test	QIAGEN therascreen EGFR RGQ mutation test
Platform	**Real time-PCR**	**Real time-PCR**
Instrument	Cobas z 480 Analyzer	Rotor-Gene® Q MDx
IVD, CDx Label	*EGFR*_CDx (US-FDA approved)	*EGFR*_CDx (US-FDA approved)
Drug	TARCEVA® (Erlotinib), TAGRISSO™ (Osimertinib)	GILOTRIF® (Afatinib)
Intended use	The test is intended to be used as an aid in selecting patients with NSCLC for whom TARCEVA® (erlotinib) and TAGRISSO™ (Osimertinib), an *EGFR* tyrosine kinase inhibitor (TKI), is indicated.	The test is intended to be used to select patients with NSCLC for whom GILOTRIF™ (afatinib), an *EGFR* tyrosine kinase inhibitor (TKI), is indicated.
Mutation coverage	G719X, E19del, T790M, S768I, E20Ins, L858R, L861Q	E19del, L858R(CDx) + L681Q, G719X, S768I, exon 20 insertions, and T790M
	Total: 42 mutation sites	Total: 15 mutation sites
Detection method	Allele-specific primer	ARMS and Scorpions
Input DNA	FFPE : 50 ng / well	FFPE : 80 ng / well
	cfDNA : 25 µl / well	-
Reaction (well) no.	3	8
Sensitivity	FFPE : 5%	FFPE : 1%
	cfDNA : <100 copies /ml	-
Application	FFPE, cfDNA	FFPE
Running time	~2 h	~3 h

Table 3. Two commercial kits for *EGFR* mutation testing

3. DNA damage in FFPETs

Formalin-fixed, paraffin-embedded tissue (FFPET) is the most widely available material for molecular diagnostics and clinical research (51). Fixing cancer tissues in buffered formalin is a standard procedure because it preserves tissue and cellular morphology prior to examination by anatomical pathologists. The routine formalin-fixation process stabilizes proteins by cross-linking, thereby maintaining tissues in an excellent condition for histopathological analysis (52). FFPET samples can be stored at room temperature, which means that damage caused by freezing can be avoided. However, the FFPET fixation procedure and long-term storage at room temperature causes several kinds of damage to nucleic acids, creating challenges to molecular analyses using FFPET-derived DNA (FFPET-DNA) (Fig. 3) (53).

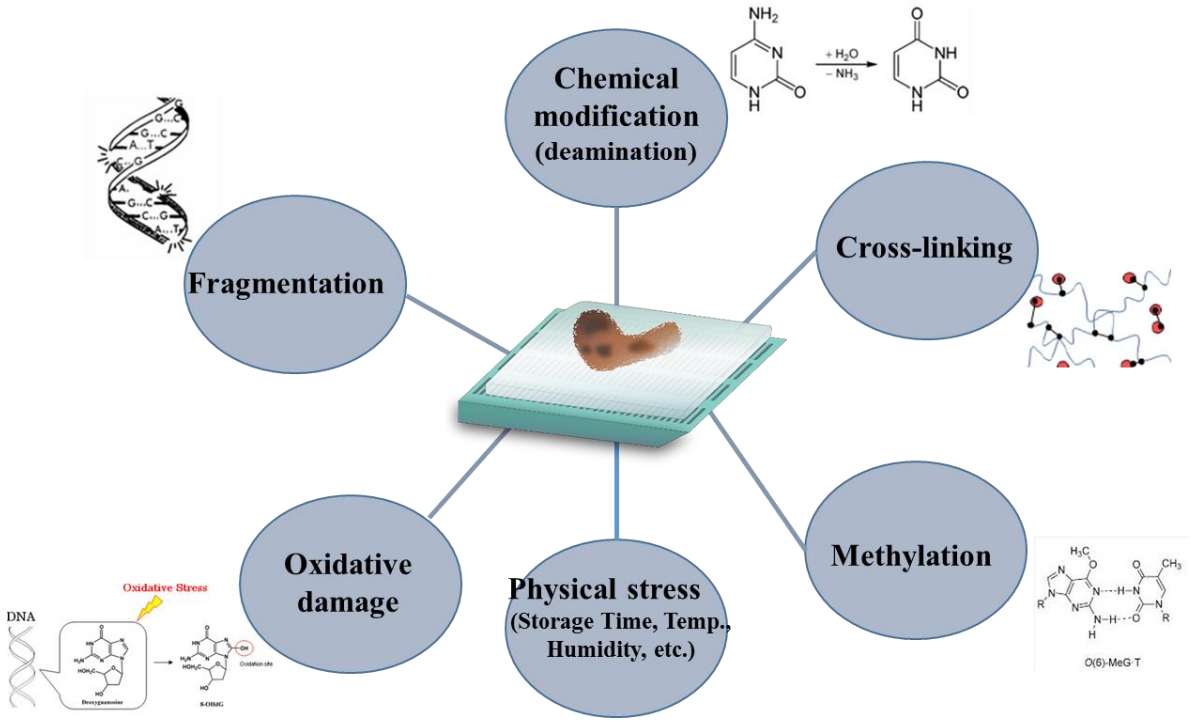


Figure 3. Various DNA damage in FFPET. The FFPETs have some advantages, such as easier storage, but it is well known that formalin fixation results in DNA damage.

3.1 DNA fragmentation

Increased storage time and low pH lead to fragmentation of DNA in FFPET. PCR performed by using fresh FFPET is usually much more successful than that of using FFPET that has been stored for some time, indicating that DNA fragmentation occurs continuously during storage (54-57). Fragmented DNAs reduce the success of PCR amplification since this depends on the size of the amplicon (58). Also, DNA fragmentation correlates inversely with sequence coverage and can lead to substantial changes in the amount of amplifiable template, which is in turn related to the presence of sequencing artifacts (57, 59).

3.2 Hydrolytic deamination

Hydrolytic deamination of cytosine residues yields uracil lesions, which are a major source of artifacts when sequencing FFPET-DNA (60-62). In the human genome, cytosine deamination occurs at an estimated rate of 60–500 events/day (63). Among the sequence artifacts detected in FFPET DNA, C:G > T:A transitions are the most common type of single nucleotide variant (Fig. 4) (64). Recent comparisons of sequencing data generated from fresh-frozen and FFPET demonstrated that the majority of false-positive and -negative mutation calls were made for FFPET samples, particularly in low quality samples (64, 65). Recently, Do, H *et al*. have examined sequence artifacts in FFPET DNA samples, highlighting the importance of DNA lesions (66). For example, the T790M mutation in *EGFR* is a predictor of TKI resistance in NSCLC patients (67); therefore, an FFPET artefact causing a false-positive result may lead to a clinician selecting the wrong treatment option (68, 69).

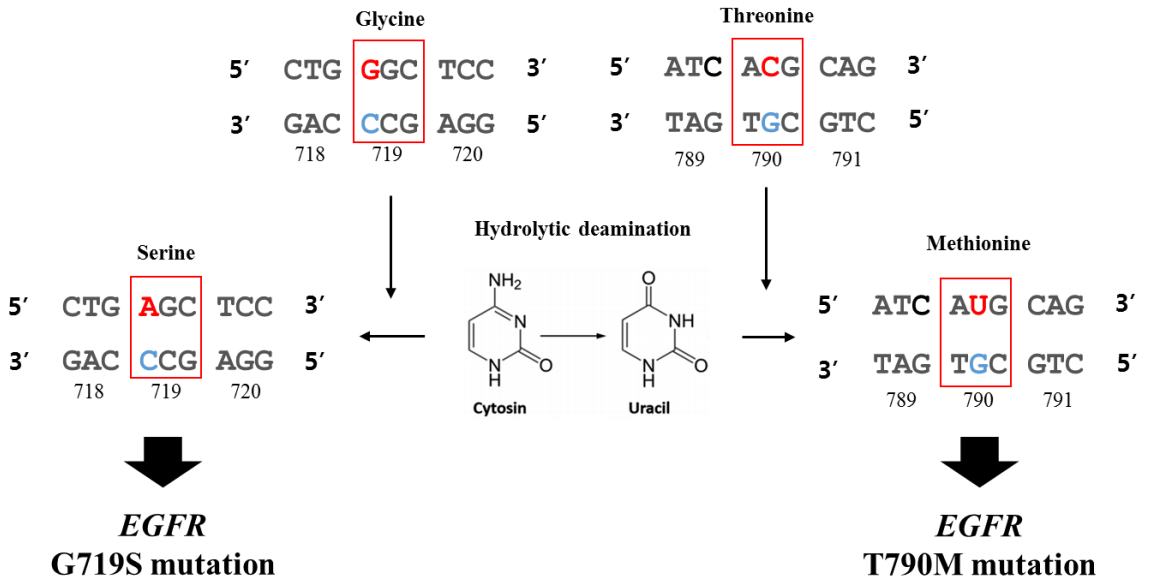


Figure 4. Hydrolytic deamination of cytosine residues. Deamination of cytosine residues results in non-reproducible artifacts. *EGFR* G719S and T790M are the most common false-positive results called after deamination.

3.3 Formaldehyde-induced abasic site

Formaldehyde induced abasic sites are generated by depurination. When it reacts with oxygen in the atmosphere, formaldehyde is readily oxidized to formic acid, which reduces the pH of a formalin solution. Formalin solutions are therefore buffered to maintain a neutral pH. In DNA, the *N*-glycosidic bonds between a purine base and its deoxyribose moiety are most susceptible to hydrolysis at low pH (70); thus, fixation of tissues in unbuffered formalin will lead to a marked reduction in the amount of amplifiable DNA template (71). Abasic sites also cause problems with respect to sequence analysis. Chemical alterations in DNA block the passage of DNA polymerase, resulting in sequence artifacts (72, 73).

3.4 Formaldehyde-induced cross-link

Cross-linking reduces the stability of double stranded DNA, which in turn affects the amount of FFPET DNA that can be amplified by PCR (74). Formaldehyde, the main component of formalin, induces crosslinks between proteins and between proteins and DNA; it also induces DNA-formaldehyde adducts and inter-strand DNA crosslinks, all of which have a primary genotoxic effect (75-77). Cross-linking not only causes problems in terms of DNA isolation, but also reduces PCR amplification efficiency (25). Therefore, formaldehyde-induced cross-linking reduces the overall stability of DNA (78), which makes downstream analysis difficult.

3.5 DNA damage during preparation of FFPE blocks (physical stress)

As mentioned above, formalin fixation has a time-dependent adverse effect on the quality of FFPET DNA and subsequent PCR results (79). Because different laboratories use different

fixation protocols and store tissues for different times, the quality of FFPET DNA should be checked prior to its use in clinical studies. Other than storage duration and fixation procedures, fixation time is another critical factor for PCR analysis. Indeed, Inoue et al. revealed that the quality of DNA in FFPET tissues varied markedly depending on the fixation time (79). It is imperative that all nucleic acid assays used for clinical purposes use properly prepared and stored FFPE tissues. However, many hospitals lack appropriate FFPET storage facilities, resulting in numerous unqualifiable samples (80). Thus, for successful assay in the clinic, DNA quality is paramount. Furthermore, prior to the use of FFPET samples aged more than 10 years for retrospective studies, the quality of the DNA should be tested vigorously.

3.6 Strategies for minimizing sequence artifact

Minimizing sequence artifacts is critical for accurate detection of mutations in FFPET DNA. Accurate detection of EGFR-TKI actionable and/or resistance mutations enables identification of patients who will benefit from targeted therapy and avoid adverse effects associated with unnecessary treatment of non-responsive patients. Several strategies can be used to minimize DNA damage, which is the main limitation for any PCR-based assay and/or amplicon sequencing-based technique. Several papers describe strategies for minimizing sequence artifacts (Table. 4) (66, 81).

First, fragmentation of FFPET DNA reduces the amount of amplifiable template available. As mentioned earlier, the success rate of PCR is related to amplicon size. Therefore, it is important to consider short amplicons when designing primers and probes (82, 83). In addition, the design of shorter amplicons as well as more stricter conditions for PCR amplification could be a useful way to lower the amount of unspecific PCR products, which generate of high background (81). Moreover, careful design of primers and probes are necessary to prevent non-specific artefacts such as primer dimers and chimeric products.

Second, C:G>T:A artifacts are a common form of damage found in FFPET DNA (84). *In-vitro* removal of uracil bases from FFPET DNA using uracil-DNA glycosylase (UDG) before PCR amplification reduces this problem (84). Detection of true mutations is not affected by UDG treatment. UDG is an evolutionarily conserved DNA repair enzyme that imitates the base excision repair pathway and removes uracil from DNA (85). Thus, pre-treatment with UDG is a simple and effective strategy for reducing C:G>T:A artefacts.

Third, reversal of formaldehyde-induced DNA-DNA and DNA-protein crosslinks by heating samples to over 90℃ increases the amount of amplifiable DNA template (82, 86-88).

Lastly, template quantity and quality are crucial for optimizing assay conditions and accurate detection of mutations. The same measured quantity of different FFPET DNAs can contain different amounts of amplifiable template, depending on the degree of DNA fragmentation (59). Spectrophotometric and fluorometric methods tend to seriously overestimate the actual amount of amplifiable FFPET-DNA (69). Thus, PCR-based methods such as qPCR and digital (d)PCR are more suitable for quantifying the amount of actual amplifiable FFPET DNA.

Damage	Strategy
Fragmentation	1. Design shorter amplicons 2. Assessment of DNA integrity 3. Quantification of amplifiable templates using PCR
Deamination	1. Pre-treatment with UDG
Abasic sites	1. Use of specific DNA polymerases (e.g. *pfu* polymerase) that have low bypass efficiency over DNA lesions such as uracil and abasic sites 1. Use of a high-fidelity DNA polymerase to reduce polymerase errors
Cross-linking	1. Heat treatment of remove formaldehyde-induced crosslinks 2. Extended proteinase K treatment to digest tissues and remove proteins cross-linked to DNA
Physical stress	1. Establishment of SOPs for sample storage conditions 2. Establishment of SOPs for fixation procedures

Table 4. Strategies for minimizing sequence artifacts in FFPET-DNA.

4. Liquid biopsy

Direct testing of tumor tissue is used widely to detect gene mutations. However, methods used to determine mutation status in tumor tissue from patients with NSCLC have limitations (89). First, tumor samples are "contaminated" by normal tissue. Second, the biopsy may not represent the total burden of mutated cells, particularly in patients with metastatic disease. Third, the quality of extracted and stored DNA is variable; poor quality DNA may prevent accurate analyses. Also, patients with progressive disease cannot always be subjected to invasive procedures. In light of these limitations, new ways of monitoring tumor genetics and tumor dynamics have been developed. Recently, cell-free circulating tumor DNA (cfDNA and ctDNA) has attracted much attention as a potential tumor marker.

Analysis of "liquid biopsies" is a rapidly expanding field of cancer research because it can be useful for diagnosis and treatment of cancer patients, as well as its non-invasive and convenience. Analysis of liquid biopsies enables [a] early diagnosis, [b] estimation of the risk of cancer recurrence and metastasis (prognostic information), [c] observation and real-time monitoring of treatment responses, [d] better understanding of the therapeutic target and mechanism(s) of resistance, and [e] better understanding of metastasis (90).

However, it is unclear whether ctDNA in liquid biopsies can be used for non-invasive assessment of EGFR mutations in NSCLC patients. The first blood-based *EGFR* mutation analysis method to be developed was based on plasma obtained from pre-treatment NSCLC patients with gefitinib using the amplification refractory mutation system (ARMS)-based *EGFR* mutation detection kit. The *EGFR* mutation status derived from tumor tissue agreed with the results from matched plasma samples in 73% of cases (91). This result showed that it was possible to use ctDNA to detect *EGFR* mutations; other studies have also used various

serum- or plasma-based methods to assess *EGFR* mutation status (92-97). A previous paper reported the application of non-invasive procedures to monitor the *EGFR* T790M (98). Monitoring the mutation by using plasma in patients gives benefit to patients by suggesting proper treatment.

Because ctDNA analysis does not involve formaldehyde fixation, the number of false-positive results (caused by deamination) is low (99). Also, the amount of ctDNA in the plasma of healthy controls is much lower than that in NSCLC patients (100, 101). However, it is very difficult to analyze ctDNA from NSCLC patient due to the low amount of absolute ctDNA. The frequent fragmentation is another obstacle for analysis. It is known that double-stranded DNA fragmentations frequently occur in the serum or plasma of cancer patients (102). Also, high levels of DNA derived from tissues of non-tumor origin make detection of ctDNA technically difficult. Since ctDNA is derived directly from the tumor, it must be quantified accurately and with high sensitivity (103-108). Due to the varying quantity and quality of DNA derived from tumors, high sensitive and specialized equipment is required for ctDNA detection. Cutting-edge technologies can be used to detect somatic mutations, loss of heterozygotes, and mutation of tumor associated genes, at low frequencies as low as 0.01% (109-113). Although several methods can be used to analyze mutations in ctDNA [e.g., digital polymerase chain reaction (dPCR) (114), mutant-enriched PCR, and peptide nucleic acid-locked nucleic acid PCR], few have been approved for ctDNA analysis.

5. Third generation PCR: Droplet digital PCR (ddPCR)

Real-time quantitative PCR (qPCR) is the most common method used to quantify expression of target genes (115, 116). However, either external calibrators or normalization to endogenous controls is required to estimate the concentration of an unknown (117). Imperfect amplification efficiency affects C_T values, which in turn limits the accuracy of this technique for absolute quantitation. ddPCR is an assay that combines state-of-the-art microfluidics technology with TaqMan-based PCR to achieve precise identification of target DNA with high sensitivity and specificity (Fig. 5, Table. 5) (118). This is because quantitation is achieved without the need for standard assays, allowing easy interpretation and an unambiguous digital readout. For ddPCR, a sample is fractionated into 20,000 droplets and PCR amplification of the template molecule(s) occurs in each individual droplet, effectively avoiding PCR bias, increasing the signal to noise ratio, removing the need for calibration or a reference, and providing absolute quantitation of a sample (117, 119). Hence, ddPCR is far simpler, faster, and less error prone than qPCR (120). Due to its technological advantages, which enable highly sensitive detection of mutations, the method has been adopted for clinical research (97, 119, 121-123). Notably, DNA integrity can also be assessed using ddPCR technology (119).

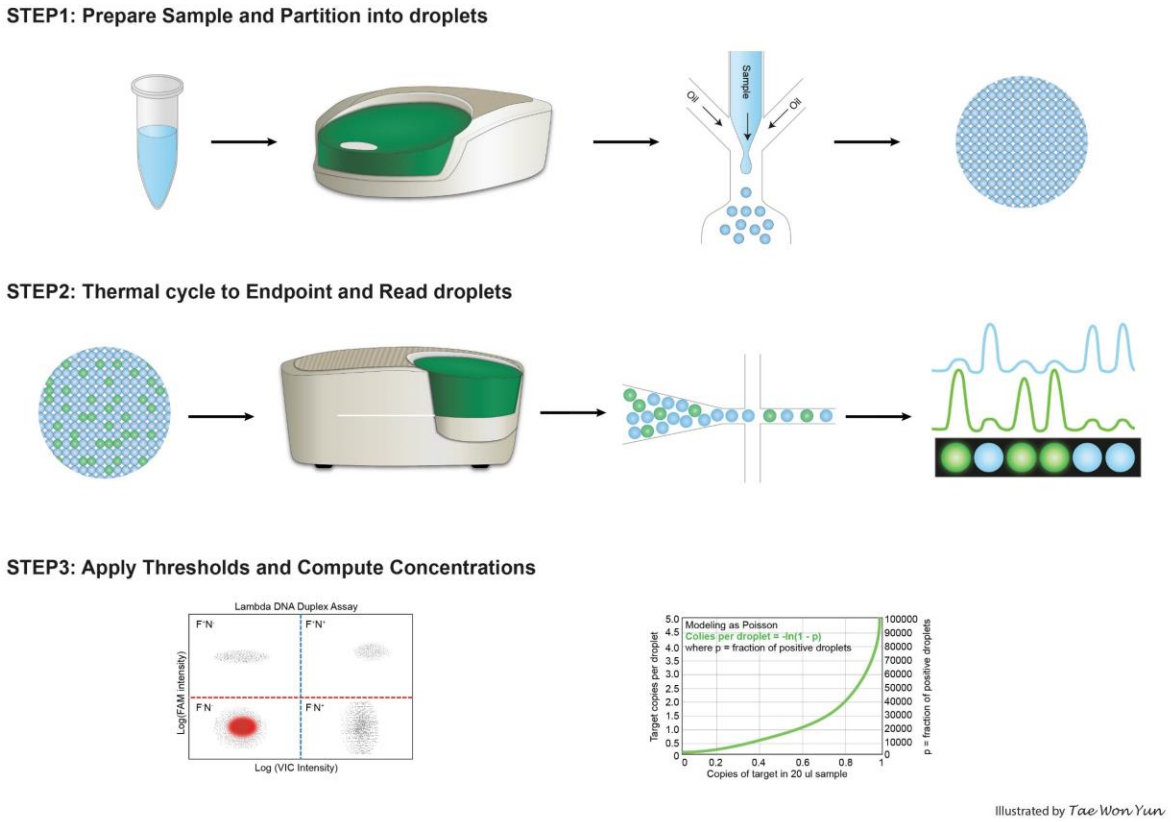


Figure 5. Schematic showing the ddPCR workflow. (A) Sample preparation and droplet generation. (B) Thermal cycling to endpoint and reading of each droplet. (C) Data analysis.

	Real time-PCR	ddPCR
Advantage	1. High throughput, routine experiments 2. Relative quantification 3. Short run time (~2 h / 96 samples) 4. Low running cost	1. High Accuracy, Precision, Sensitivity (reduced error rate, 1.2-fold ↓) 2. Relative and absolute quantification 3. Simplified quantification : neither calibration standards nor a reference is required 4. Removal of PCR bias 5. Superior partitioning : 20,000 droplets per 20 μl → higher accuracy 6. Sensitive detection of molecules with low-expression
Disadvantage	1. Dependent on C_T value of calibration standards 2. Inhibitor-dependent PCR efficiency	1. Long run time (6 h / 96 samples) 2. Higher running costs 3. Requires attention compared to qPCR

Table 5. qPCR versus ddPCR. Advantages and disadvantages of each assay.

6. Limit of blank, limit of detection and limit of quantitation

In the field of diagnostics, the ability to detect slight alterations in DNA is very important. This is known as sensitivity. Sensitivity is an important characteristic of any diagnostic tool because the ability to detect low level mutations increases the reliability of the assay. Sensitivity, limit of blank (LoB), limit of detection (LoD), and limit of quantitation (LoQ) are related by means of describing the lowest concentration of analytes (Fig. 6).

6.1 Limit of blank (LoB)

LoB is used when testing the samples that contain no analyte. It is a measure of a sample's expected maximum analyte concentration. Even though the samples lack analytes, the blank might contain low concentration of analytes. This is important when using very sensitive methods such as ddPCR. The LoB in ddPCR rules out false-positives. Therefore, it is very important to measure the LoB using standard guidelines.

LoB is estimated by blank sample's mean result and standard deviation (SD). The LoB is calculated according to:

$$LoB = \text{mean}_{blank} + 1.645\,(SD_{blank})$$

6.2 Limit of detection (LoD)

LoD is the lowest analyte concentration and it is reliable. Opposite to LoB, which measures the highest concentration of analytes, LoD measures the lowest analyte concentration. LoD is an interval where analytes are detected while performing serial dilution. The LoD is

calculated by mean and SD of the low concentration of sample. The LoD is calculated according to:

$$LoD = LoB + 1.645\ (SD_{\text{low concentration sample}})$$

6.3 Limit of quantitation (LoQ)

LoQ is not only an analyte that can be reliably detected, but it is also the lowest concentration indicator for functional sensitivity. The LoQ value can be greater or equal to the LoD, even though the LoQ and LoD are calculated independently. In clinical trials, the LoQ indicates the minimal sensitivity to drug.

The Clinical and Laboratory Standards Institute (CLSI) have published standard guidelines for determining the LoB, LoD, and LoQ (CLSI document EP17. Wayne, PA USA: CLSI; 2004). It is important to characterize the analytical performance of diagnostics tests to understand their capabilities and limitations, and to ensure they are “fit for purpose”.

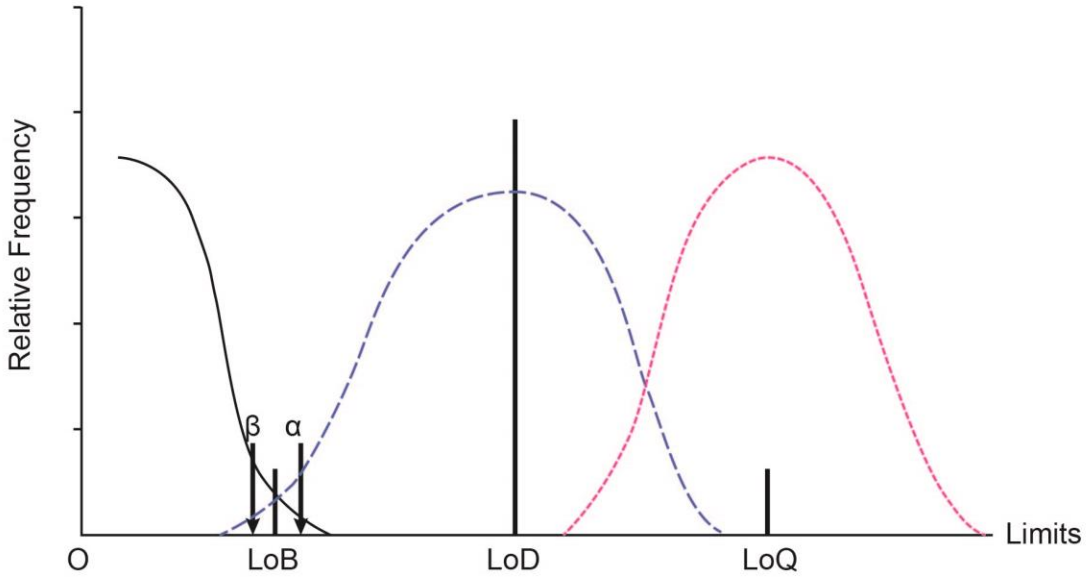


Figure 6. Relationship between the LoB, LoD, and LoQ. Distribution of replicates of a blank sample (solid black curve) and a specimen of low concentration (blue dashed curve). The LoB excludes a small proportion of blank results ("α", Type I error). The LoD is then set such that only a small proportion ("β", Type II error) of these results will fall below the LoB. A sample measurement result exceeding the LoD threshold would represent quantitative detection of the measurand (analyte) (red dotted curve).

II. Purpose of the study

Advances in science and technology have opened up new avenues in terms of molecular targeted therapy for cancer patients. For instance, several signal transduction pathways are activated in various cancers, which can be targeted by small molecules. Mutation analysis is a key factor for stratifying cancer patients according to the likely benefit of molecular targeted therapy. Therefore, accurate detection of mutations that may aid/retard cancer therapy is essential.

Imprecise mutation analysis caused by DNA damage is a very serious problem in many diagnostic fields. In particular, DNA fragmentation reduces the amount of amplifiable DNA in a tissue; this makes estimation of the mutation frequency value (based on the amount of input DNA) less accurate. FFPET is the most widely used resource for mutation analysis. But, artifactual sequence variants arising from DNA damage will be detected more frequently because stochastic enrichment (in the context of low copy targets) increases the risk of false-positives. DNA extracted from FFPET is subject to extensive fragmentation, which reduces data quality. None of the traditional tools used to check DNA integrity (e.g., UV absorbance or gel electrophoresis) actually measure the amount of amplifiable DNA or the amount of DNA fragmentation. Therefore, information on the PCR amplifiable templates will enable determining how reliable the accuracy of detected gene mutation.

Here, we report development of sample criteria to ensure minimum FFPET DNA quality for PCR. We then apply these criteria to a ddPCR-based EGFR mutation test. To establish the sample criteria, we first collected and analyzed 316 NSCLC FFPET DNA samples of various age and quality from three different sites (hospitals). We also conducted independent retrospective clinical studies using 228 NSCLC FFPET samples to verify the clinical implications of the established sample criteria. In addition, we compared the performance of the GenesWell™ ddEGFR mutation test (ddEGFR test) with that of the Cobas® EGFR mutation

test (Cobas EGFR test) in a pre-clinical and clinical studies based on 544 NSCLC FFPET samples.

The major study objectives were 1) to establish sample criteria to determine the minimum DNA quality suitable for PCR, 2) to compare the clinical performances of the ddEGFR and cobas EGFR tests.

III. Materials and Methods

1. Study design

To establish sample criteria, a total of 316 samples obtained from NSCLC patients were tested for *EGFR* mutations. A post-hoc analysis of these pre-clinical data was conducted for both the ddEGFR and cobas EGFR test results (Fig. 7). Based on the established sample criteria, an independent retrospective comparison study was performed to estimate the concordance between the ddEGFR and cobas EGFR tests; for this purpose, 228 FFPET-DNA samples from NSCLC patients were analyzed by both tests (Fig. 8). Both EGFR mutation tests were performed in a double-blind fashion by an independent laboratory (Abion Inc., Seoul, Korea). The study design (workflow) is depicted in Figure 7, 8.

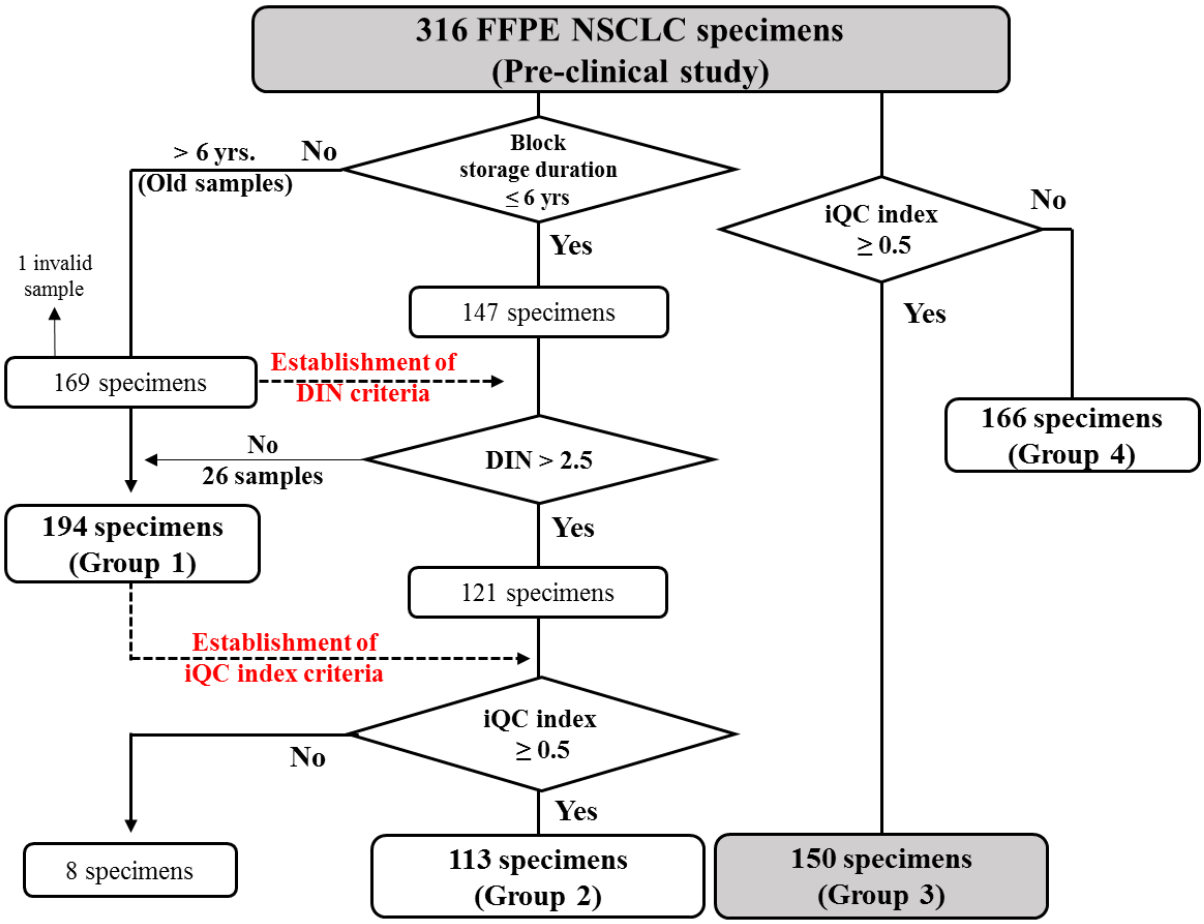


Figure 7. Study design and specimen selection (work flow of pre-clinical study). Patient sample dispositions for the applied sample criteria. For the pre-clinical study group, a total of 316 FFPET specimens were subjected to post-hoc analysis.

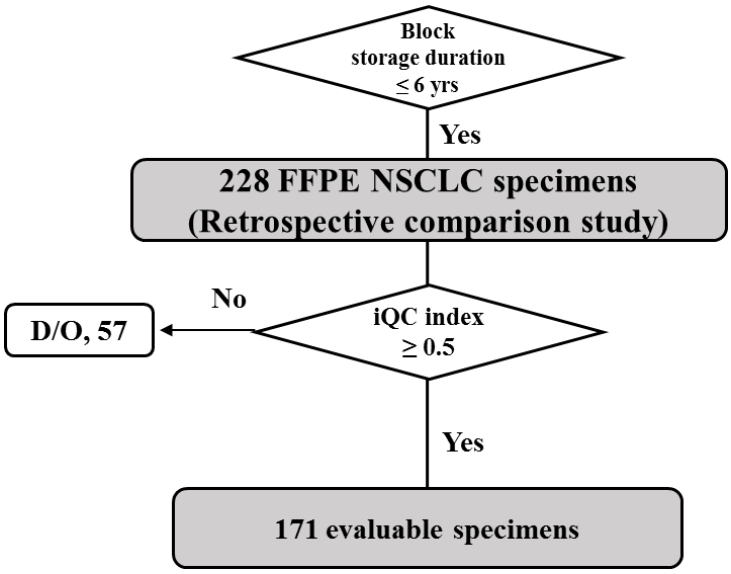


Figure 8. Study design and specimen selection (work flow of retrospective comparison study). Patient sample dispositions for the applied iQC index. For the retrospective comparison study group, a total of 228 FFPET specimens were analyzed (D/O, Drop Out).

2. FFPET collection

FFPET blocks of resected or biopsy samples from NSCLC patients (*n* = 316) collected from 2005 to 2014 were retrieved from the Department of Pathology, Samsung Medical Center (*n = 200*) (SMC; Seoul, Korea), Asan Medical Center (*n = 66*) (AMC; Seoul, Korea), and Severance Hospital (*n = 50*) (Seoul, Korea). This study was approved by the Institutional Review Board (IRB) of SMC and Seoul National University (study ID: SMC-2014-05-084-002). For the retrospective comparison study, 228 archived FFPET blocks from NSCLC patients collected between 2010 and 2016 were obtained from Department of Pathology, SMC. This study was approved by the IRB of SMC and the Ministry of Food and Drug Safety (MFDS) of Korea (study ID: SMC-2016-07-104-002). From each FFPET, 10 μm sections were cut and subjected to DNA extraction. H&E-stained sections containing tumor lesions marked by a pathologist (S.W.C.) were scanned, and the cancer/normal (C/N) ratio was calculated using the Pannoramic Viewer Software v.1.15.4 (3DHISTECH, Budapest, Hungary). Patient information was anonymized and de-identified prior to analysis.

3. DNA extraction and determination of DNA quantity and quality

DNA extraction from FFPETs was performed using an automated Tissue Preparation System (TPS; Siemens Healthcare, Erlangen, Germany) with the VERSANT® Tissue Preparation Reagents, as described previously (124). Total nucleic acids were eluted with 100 μL elution buffer containing UDG provided by the manufacturer. For all samples, DNA concentration was assessed using the Qubit™ 3.0 Fluorometer and Qubit dsDNA BR assay kit (ThermoFisher Scientific, MA, USA). The DNA integrity number (DIN), reflecting the DNA fragmentation level (125) of genomic DNA (gDNA), was analyzed on a 2200 TapeStation system with

Genomic DNA Screen Tape (Agilent Technologies, CA, USA). The samples were prepared by mixing 1 μl of DNA sample with 10 ul of genomic DNA sample buffer. A genomic DNA ladder was placed in the first lane of 8-strip tube followed by the samples. The prepared strip tube was mixed and placed in the 2200 TapeStation instrument. Samples were analyzed using TapeStation Analysis v.01.15 Software.

4. Validation of internal quality control (iQC) of ddEGFR test

The ddEGFR test (Gencurix Inc., Seoul, Korea) was designed as a highly sensitive ddPCR-based diagnostic test for detecting 45 mutation sites within the exon 18–21 region of the *EGFR* gene using four reactions. The amplified fragments, which contain the fluorophores FAM™ or HEX™, are displayed as dots (droplets) and can be used to calculate concentrations (copies/20 μL) based on the Poisson distribution (118). The details of specific mutations detected by the assay are provided in Table 6 and Table 7.

No.	Target exon	COSMIC No. *	Target mutant A.a	Target mutant site	Result
1		6239	p.G719A	c.2156G>C	
2	**Exon18**	6252	p.G719S	c.2155G>A	**G719X**
3		6253	p.G719C	c.2155G>T	
4		6223	p.E746_A750del	c.2235_2249 del 15	
5		13551	p.E746_T751>I	c.2235_2252 > AAT	
6		12728	p.E746_T751del	c.2236_2253 del 18	
7		12678	p.E746_T751>A	c.2237_2251 del 15	
8		12367	p.E746_S752>A	c.2237_2254 del 18	
9		12384	p.E746_S752>V	c.2237_2255>T	
10		6225	p.E746_A750del	c.2236_2250 del 15	
11		6220	p.E746_S752>D	c.2238_2255 del 18	
12		13550	p.E746_A750>IP	c.2235_2248>AATTC	
13		12403	p.L747_S752>Q	c.2239_2256>CAA	
14		12422	p.L747_A750>P	c.2238_2248 >GC	
15		12419	p.L747_T751>Q	c.2238_2252 >GCA	
16		6218	p.L747_E749del	c.2239_2247 del 9	
17		12382	p.L747_ A750>P	c.2239_2248 TTAAGAGAAG>C	
18	**Exon19**	6210	p.L747_T751>S	c.2240_2251 del 12	**19del**
19		12383	p.L747_T751>P	c.2239_2251>C	
20		13552	p.E746_T751>IP	c.2235_2251>AATTC	
21		6254	p.L747_T751del	c.2239_2253 del 15	
22		6255	p.L747_S752del	c.2239_2256 del 18	
23		12387	p.L747_P753>Q	c.2239_2258 >CA	
24		12370	p.L747_P753>S	c.2240_2257 del 18	
25		12416	p.E746_T751>VA	c.2237_2253>TTGCT	
26		-	-	c.2239_2257>GT	
27		26038	p.K745_E749del	c.2233_2247del15	
28		13556	p.S752_I759del	c.2253_2276del24	
29		12386	p.E746_T751>V	c.2237_2252>T	
30		12385	p.E746_S752>I	c.2235_2255>AAT	
31		18427	p.E746_P753>VS	c.2237_2257>TCT	
32		12369	p.L747_T751del	c.2240_2254 del 15	
33		23571	p.L747_T751delLREAT	c.2238_2252 del 15	
34		12376	p.V769_D770insASV	c.2307_2308 insGCCAGCGTG	
35		12377	p.H773_V774insH	c.2319_2320 insCAC	
36	**Exon20**	12378	p.D770_N771insG	c.2310_2311 insGGT	**E20Ins**
37		13428	p.D770_N771insSVD	c.2311_2312insGCGTGGACA	
38		13558	p.V769_D770insASV	c.2309_2310AC>CCAGCGTGGAT	
39		6241	p.S768I	c.2303G>T	**S768I**
40	**Exon20**	6240	p.T790M	c.2369C>T	**T790M**
41		-	p.C797S	c.2389T>A	**C797S**

42		-	p.C797S	c.2390G>C	
43	**Exon21**	6224	p.L858R	c.2573T>G	**L858R**
44		12429	p.L858R	c.2573_2574TG>GT	
45		6213	p.L861Q	c.2582T>A	**L861Q**

* Catalogue of Somatic Mutations in Cancer (COSMIC). 2016, v.78.

http://cancer.sanger.ac.uk/cosmic

Table 6. Forty-five *EGFR* mutations detected by the ddEGFR mutation test.

The non-clinical performance studies followed the guidelines approved by the Clinical and Laboratory Standards Institute (CLSI) and the Korea-MFDS. For validation of internal quality control of ddEGFR, FFPE reference standard DNA extracts (HDx™ Reference Standard, Horizon Discovery, Cambridge, UK) for EGFR mutations were blended with fixed amounts of wild-type gDNA (3.3 ng, 1000 GE; Promega) and each sample, with a target MI of 1.5%. In addition, four serial dilutions of each sample (9.9 ng, 6.6 ng, 3.3 ng, and 1.65 ng) were prepared and analyzed using the ddEGFR test. The iQC copies and target MI of each sample was confirmed based on the input DNA concentration and target MI (1.5%).

5. Biomarker analysis

The ddEGFR test was performed in a 20 μL volume containing 3.3 ng (1000 GE)/reaction of template DNA on a Droplet Digital™ PCR (ddPCR) system (Bio-Rad, Hercules, CA, USA). The ddPCR assay was conducted as described previously (124). Amplification conditions consisted of a 10 min activation period at 95 ℃, followed by 40 cycles of a 30 sec at 94 ℃ for denaturation, 1 min at 60 ℃ for annealing/extension, and a final 10 min inactivation step at 98 ℃. After thermal cycling, plates were transferred to a QX200 droplet reader to read the droplets using the QuantaSoft v1.7.4 software (Bio-Rad) to assess the number of droplet positive for *EGFR*. Thresholds for detection were set manually based on results from non-template control wells and negative control wells containing wild-type gDNA (Promega).

PCR amplification for the cobas EGFR test (Roche Molecular Systems Inc., Branchburg, NJ, USA) was performed on a cobas® z 480 Analyzer. The cobas EGFR test requires 150 ng total input DNA. The mutation result reporting is fully automated, mutation was reported as either G719X in exon 18, 19del in exon 19, S768I in exon 20, T790M in exon 20, exon 20

insertion, or L858R in exon 21 (126). Both mutation tests were analyzed in a double-blind fashion, and the results were matched after analysis.

For mutation screening of *EGFR* exons 18, 19, 20, and 21 by 2× bidirectional Sanger sequencing, regions of interest were amplified by PCR, and the amplified samples were processed at an independent laboratory (Macrogen, Seoul, Korea) using a validated protocol. Sanger sequencing results were cross-checked and interpreted by a pathologist (Y.L.C).

6. Methods correlation and statistical analysis

Positive percent agreement (PPA), negative percent agreement (NPA), and overall percent agreement (OPA) of the ddEGFR and cobas EGFR tests were compared in pre-clinical and retrospective comparison studies. Agreement analysis for all methods was based on mutation report calls (Table. 7). Statistical analysis was performed using GraphPad Prism™ (GraphPad Software Inc., San Diego, USA) and the R 1.6.12 package 'psych' (http://CRAN.R-project.org/package=psych). For the agreement analyses, PPA, NPA, and OPA were calculated with their corresponding 95% confidence intervals (CIs).

IV. Results

1. Validation of internal quality control (iQC) of ddEGFR test

Because the ddPCR-based test can lead to the false positive results by both its intrinsic high sensitivity and FFPET characteristics, the cut-offs of ddEGFR test were determined based on false-positive analyses using normal FFPET. Mutation calls were identified based on true-positive mutation values higher than limit of blank (LoB) and limit of detection (LoD) mutation index (MI) [1], which were established by analytical performance studies (Table. 7). MI is a numerical value representing the ratio of mutant to internal quality control (iQC) copies, calculated as follows:

$$\text{MI (\%)} = [\text{Mutant copies} / \text{iQC copies}] \times 100 \qquad [1]$$

In the ddEGFR test, iQC copies can be converted to concentration of input DNA using an FFPE reference standard, suggesting that iQC index [2] is a representative index of amplifiable DNA. Because iQC copies were analyzed using 3.3 ng (1,000 genomic equivalents [GE]) of input DNA per reaction well, iQC index was calculated as follows:

$$\text{iQC index} = [\text{iQC copies} / \text{input DNA copies}] \qquad [2]$$

An iQC index value close to “1” means that the quality of DNA is better. Figure 9 shows the concept and example of iQC index.

Using 40 wild-type FFPET samples, false-positive rates were determined for mutant calling of eight targets. The maximum number of copies was 5.4 per reaction, and the false-positive rates were below 0.5% of MI (Fig. 10).

Exon	Mutations detected	Mutation report call	LoB copies / MI (%) *	LoD MI (%) †
18	G719A, G719C, G719S	G719X	5.6 / 0.22	0.77
19	30 deletions	19del	3.0 / 0.09	0.83
20	S768I	S768I	1.5 / 0.05	0.83
	T790M	T790M	6.8 / 0.34	0.78
	C797S §	C797S	1.6 / 0.03	0.75
	5 Insertions	E20Ins	1.6 / 0.06	0.62
21	L858R	L858R	1.6 / 0.03	0.71
	L861Q	L861Q	1.4 / 0.05	0.74

* LoB = mean $_{blank}$ + 1.645 (SD $_{blank}$) (127)

† LoD MI (%) = mean $_{low\ concentration\ sample}$ + 1.645 (SD $_{low\ concentration\ sample}$)

§ using the plasmids containing the non-predominant mutation for analytical performance

Table 7. ddEGFR test coverage. The test is designed to detect G719X (G719A, G719C, and G719S) mutations in exon 18, deletions, complex mutations (S768I, T790M, and C797S) in exon 19, insertions in exon 20, and the L858R and L861Q mutations in exon 21. The LoB were calculated using 9.9 ng of normal FFPET-DNA. The LoD was measured 6 points (3-0.1%) serially diluted using the NSCLC FFPET-DNA (19del, L858R) and reference standard FFPET-DNA (the other mutations) with wild-type FFPET-DNA. The LoD was determined by the lowest amount of DNA that gave an *EGFR* "Mutation Detected" rate of at least 95% for the target mutation.

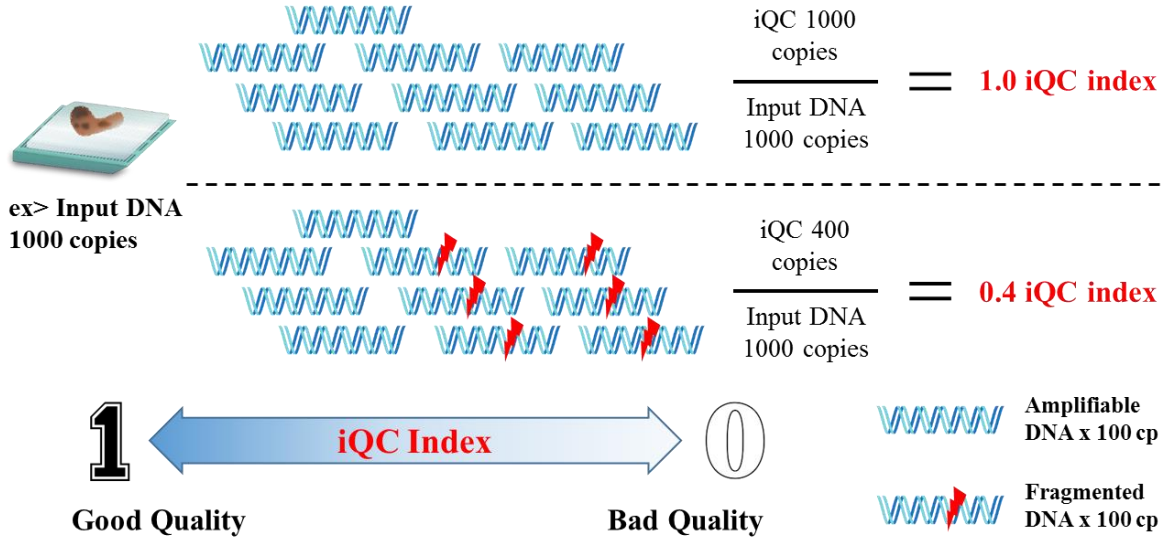
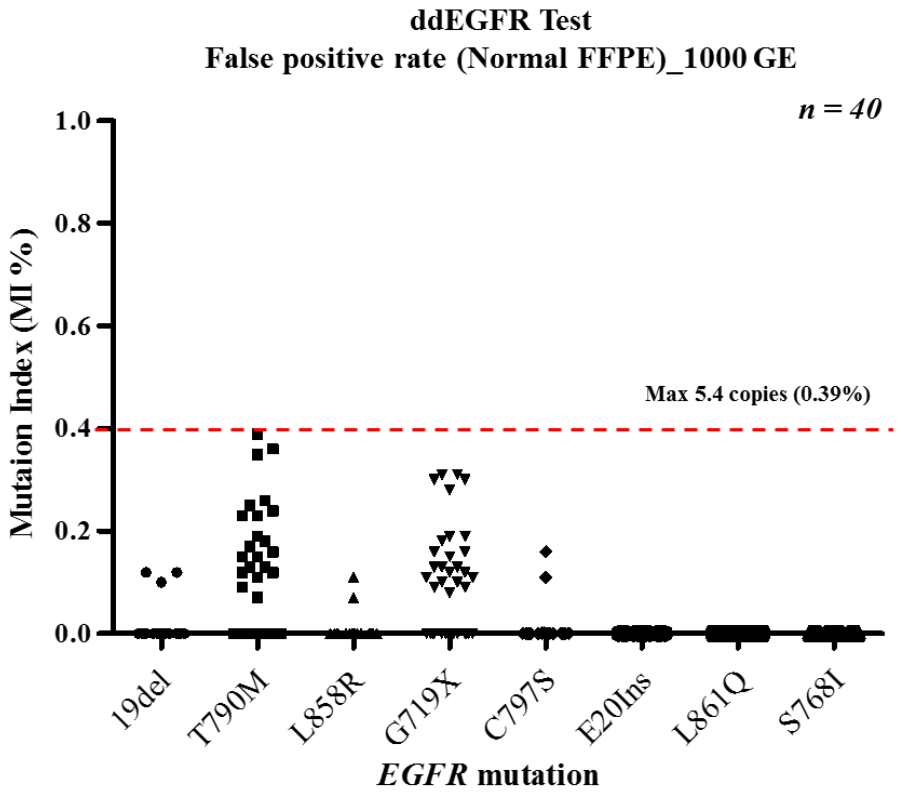


Figure 9. The concept of iQC index. iQC copy reflects the amount of amplifiable DNA. The amount of amplifiable DNA is represented as an iQC index compared to the proportion of input DNA, and the closer to "1" the better quality of DNA.



* Mutation Index (MI %) = [Mutation copies / iQC copies] x 100

Figure 10. Determination of a suitable cut-off based on false-positive analysis using normal FFPET blocks. Forty normal FFPET specimens were tested, and eight targets were evaluated for each specimen. The maximum number of copies was 5.4 per reaction (red dotted line) and the false-positive rate was below 0.5%.

We evaluated iQC using reference standard. Reference standard FFPET-DNA extract for *EGFR* mutations was blended with fixed amount of wild-type gDNA (3.3 ng, 1,000GE) targeting a 1.5% mutation level. The expected iQC index and MI were given by the calculated from the quantity of input DNA. As a result, the measured MI (%) and iQC index were observed close to match with expected value (iQC index = 1, MI = 1.5%) (Fig. 11). Moreover, iQC was also validated by serial dilution with four concentrations of reference standard FFPET-DNA, and observed that the measured values close match with the each expected values (Fig. 12) suggesting that iQC copies can represent the amount of input DNA.

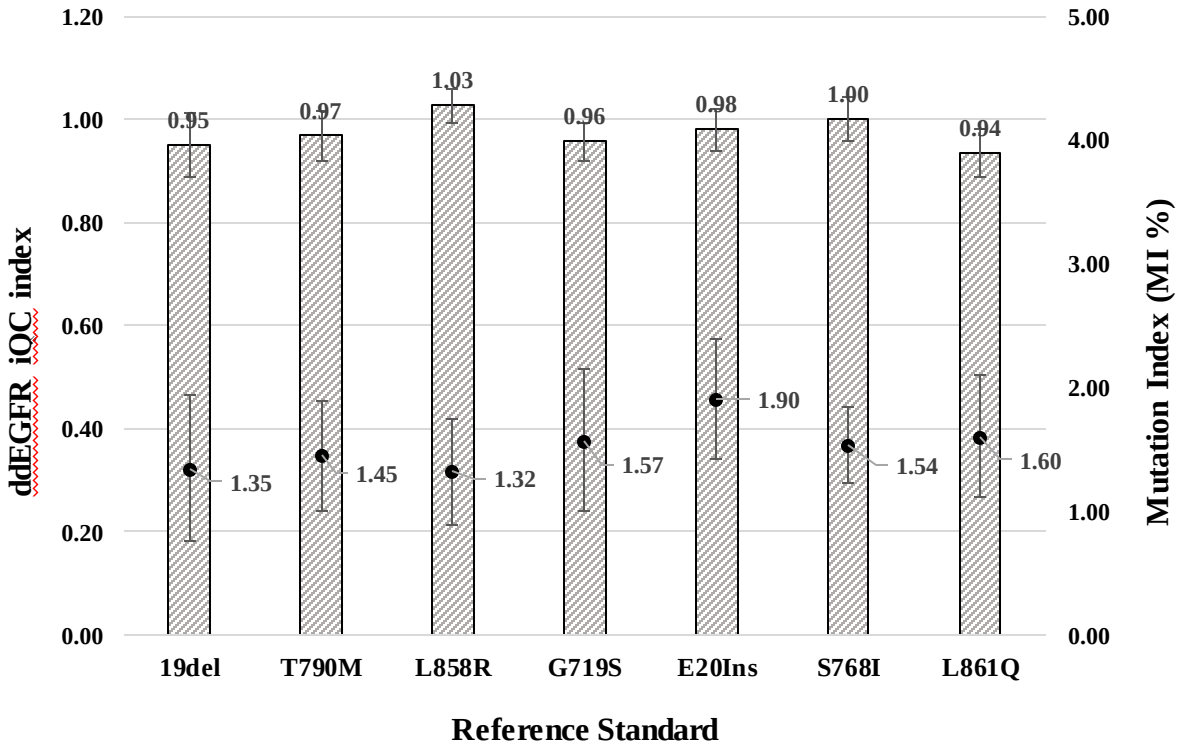


Figure 11. Validation of internal quality control of ddEGFR test (MI validation). Internal quality control (iQC) validation using reference standard. Each FFPE reference standard DNA extract for the *EGFR* mutations was blended with a fixed amount of wild-type gDNA (3.3 ng, 1000GE) targeting a 1.5% mutation level, which was validated for use in the ddEGFR test. Values are expressed as the mean ± SD of nine experiments.

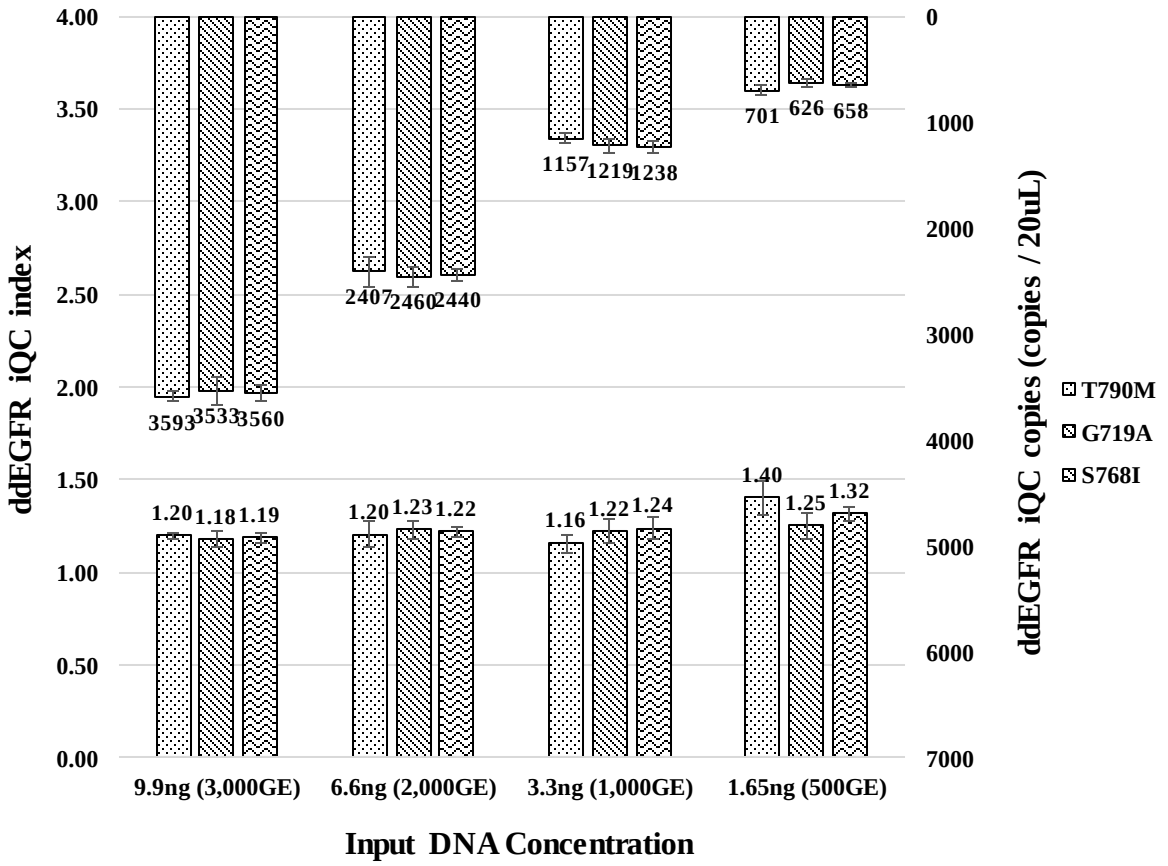


Figure 12. Validation of internal quality control of ddEGFR test (iQC copy and iQC index). Internal quality control (iQC) validation using the reference standard. Four serial dilutions of each FFPET reference standard DNA extract were prepared and subjected to the ddEGFR test. Values are expressed as the mean ± SD of three experiments.

2. Comparison of ddEGFR and cobas EGFR tests without sample criteria

The *EGFR* mutations in 316 NSCLC FFPET samples were analyzed using the ddEGFR and cobas tests. Both methods yielded valid results for all but one of the samples. Surprisingly, the ddEGFR and cobas EGFR tests were low concordant (PPA = 94.04%, NPA = 63.41%, OPA = 78.10%, kappa coefficient value (κ) = 0.6650) (Table. 8, Table. 9). The cobas EGFR test also exhibited very low concordance with Sanger sequencing of 299 samples (PPA = 59.30%, NPA = 75.00%, OPA = 65.63%, κ = 0.4526) (Table. 10, Table. 11).

All Samples (*n = 316*)		cobas EGFR Test		
		MD	**MND**	**Total**
ddEGFR Test	**MD**	139	62 *	201
	MND	9	104	113
	Total	148	166	314
PPA (95% C.I.)	94.04% (88.99−97.24%)			
NPA (95% C.I.)	63.41% (55.55−70.79%)			
OPA (95% C.I.)	78.10% (73.12−82.54%)			
PPV (95% C.I.)	70.30% (63.48−76.51%)			
NPV (95% C.I.)	92.04% (85.42−96.29%)			

* 3 samples: cobas, 19del; ddEGFR, 19del/T790M

1 sample: cobas, 19del; ddEGFR, 19del/L858R

1 sample: cobas, E20Ins; ddEGFR, E20Ins/T790M

1 sample: cobas, S768I; ddEGFR, S768I/L858R

Table 8. A total of 316 samples with valid ddEGFR and cobas EGFR test results were included in the agreement analysis. PPA, NPA, and OPA between the ddEGFR and cobas EGFR tests for the detection of *EGFR* mutations were 94.04%, 63.41%, and 78.10%, respectively.

All Samples (*n = 316*)		cobas EGFR Test														
		G719X	**19del**	**T790M**	**E20Ins**	**S768I**	**L858R**	**G719X, T790M**	**G719X, S768I**	**19del, T790M**	**19del, L858R**	**T790M, L858R**	**T790M, E20Ins**	**S768I, L858R**	**MND**	**Total**
ddEGFR Test	**G719X**	1													1	2
	19del		85												4	89
	T790M			0												0
	E20Ins				0										2	2
	S768I					0										0
	L858R		1 †				48								47	96
	G719X,T790M							0							1	1
	G719X,S768I								3							3
	19del,T790M		3							0						3
	19del,L858R		1								0					1
	T790M,L858R											0			1	1
	T790M,E20Ins				1								0			1
	S768I, L858R					1								2		3
	MND		9												104	113
	Total	1	99	0	1	1	48	0	3	0	0	0	0	2	160	315

† 1 sample was excluded from the analysis, 1 sample: Invalid

κ coefficient = 0.6650 (95% C.I. 59.98−73.03%)

Table 9. Detail concordance table of 316 samples with valid ddEGFR and cobas EGFR test.

All samples (*n = 299*)		Sanger sequencing		
		MD	**MND**	**Total**
cobas EGFR test	**MD**	102	29 *	131
	MND	70 †	87	157
	Total	172	116	288
PPA (95% C.I.)	59.30% (61.56−66.72%)			
NPA (95% C.I.)	75.00% (66.11−82.57%)			
OPA (95% C.I.)	65.63% (59.83−71.10%)			
PPV (95% C.I.)	77.86% (69.78−84.65%)			
NPV (95% C.I.)	55.41% (47.28−63.34%)			

* 1 sample: Sanger, L858R; cobas, L858R, S768I

† 4 samples: Sanger, 19del, L858R; cobas, 19del

1 sample: Sanger, 19del, L858R; cobas, L858R

Table 10. Concordance of *EGFR* mutation detection between the cobas EGFR test and Sanger sequencing, without application of exclusion criteria. A total of 299 samples with valid Sanger sequencing and cobas EGFR test results were included in the analysis. Seventeen samples could not be evaluated for concordance because Sanger sequencing did not yield results.

All samples (*n = 299*)		Sanger Sequencing														
		G719X	**19del**	**T790M**	**E20Ins**	**S768I**	**L858R**	**19del,G719X**	**19del, T790M**	**19del, L858R**	**G719X, T790M**	**G719X, S768I**	**T790M, L858R**	**S768I, L858R**	**MND**	**Total**
cobas EGFR test	**G719X**	0													1	1
	19del		62				8 §			4					18	92
	T790M			0												0
	E20Ins				0										1	1
	S768I					0										0
	L858R		2 §				40			1					5	48
	19del,G719X							0								0
	19del, T790M								0							0
	19del, L858R									0						0
	G719X,T790M										0					0
	G719X,S768I		1 §									0			2	3
	T790M,L858R												0			0
	S768I, L858R						1							0	1	2
	MND		24				27	1		13					87	152
	Total	0	89	0	0	0	76	1	0	18	0	0	0	0	115	299

§ 11 samples were excluded from the analysis

κ coefficient = 0.4526 (95% C.I. 37.45−53.07%)

Table 11. Detail concordance of *EGFR* mutation detection between the cobas EGFR test and Sanger sequencing, without application of exclusion criteria.

3. Proof-of-concept for determination of minimum DNA quality suitable for PCR using the ddPCR method

To increase the concordance between the ddEGFR and cobas EGFR tests, we investigated the minimum DNA quality suitable for PCR analysis by re-analyzing the ddEGFR data, which provided iQC copies and index (Fig. 11, 12). FFPET block storage duration was reflected in the amount of amplifiable DNA, decreasing below 50% (mean of iQC index = 0.31, Standard deviation, SD = 0.57) of 1000 GE in 7–11-year-old samples. By contrast, in 2–6-year-old samples, the amount of amplifiable DNA was around 100% (mean of iQC index = 1.07, SD = 0.69) (Fig. 13). Therefore, iQC index decreased with storage duration when FFPET was stored at room temperature. The pattern of DIN values from 315 FFPET-DNA samples measured in parallel was similar to those of iQC copies and index (Fig. 14).

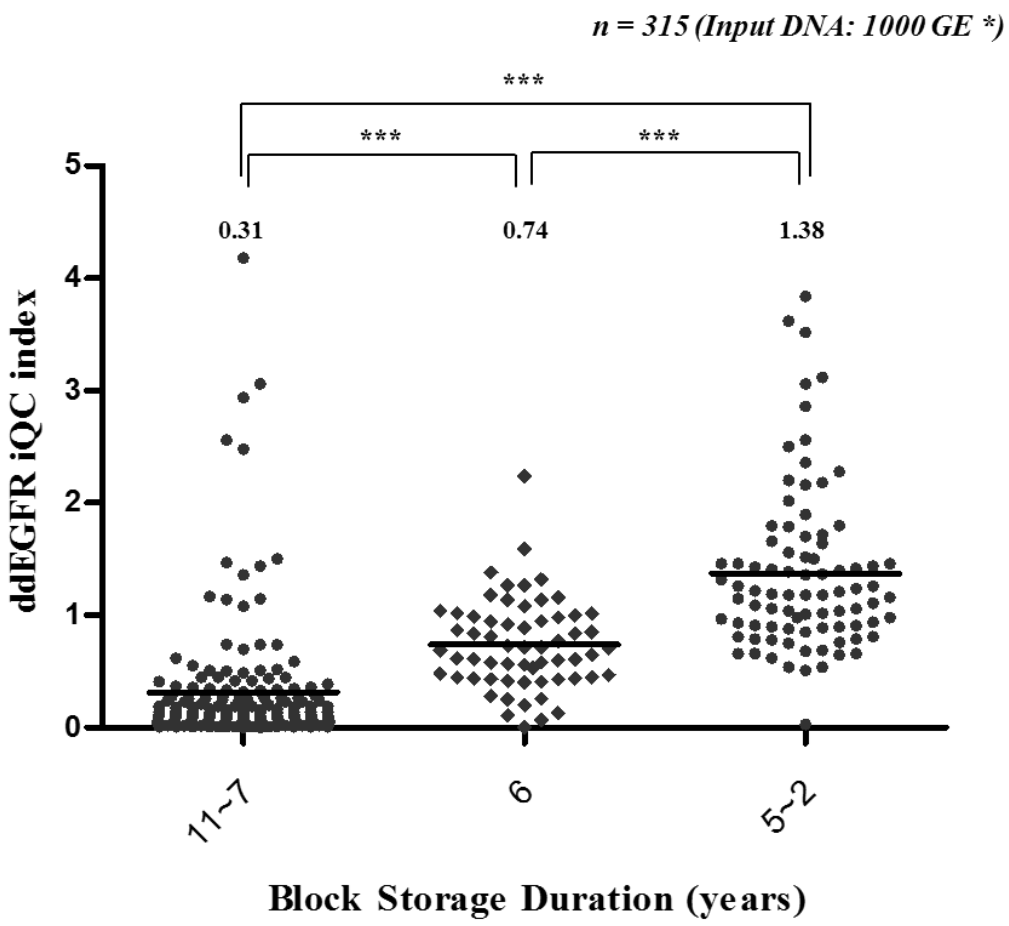


Figure 13. Distributions of ddEGFR iQC index corresponding to sample storage time. The black line indicates the median value (*Genomic Equivalence; ****p. < 0.0001*).

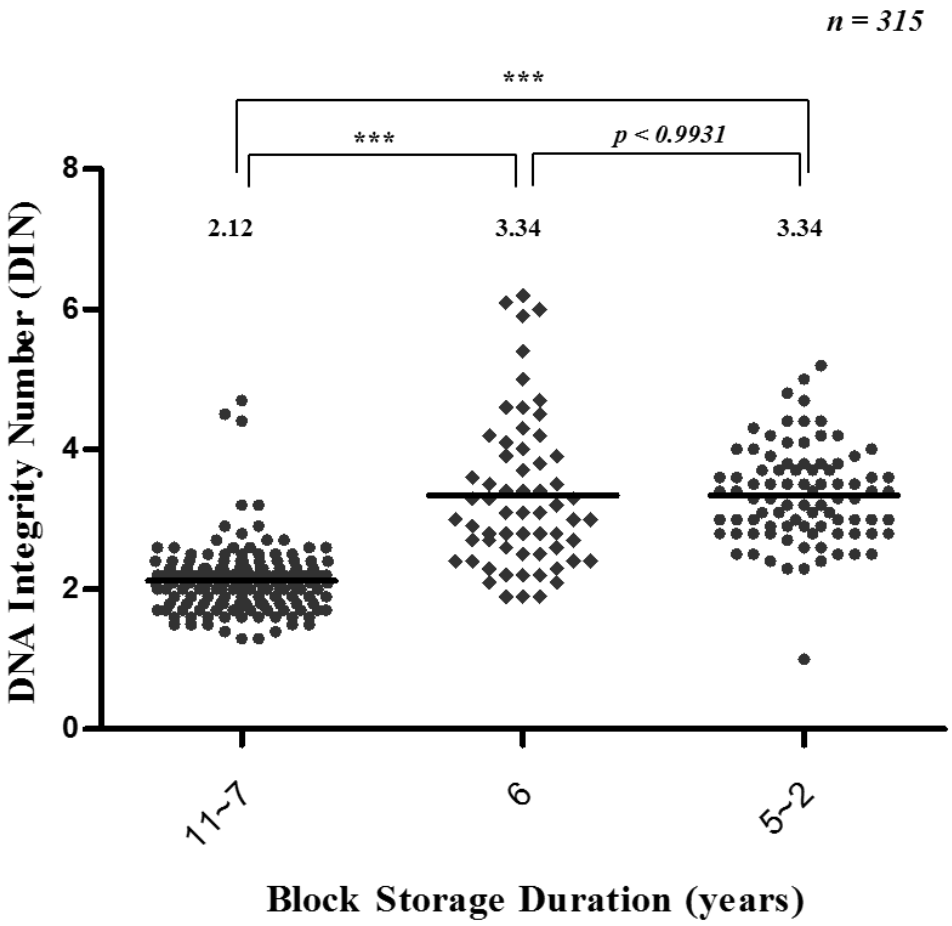


Figure 14. Distributions of DIN value corresponding to sample storage time. The black line indicates the median value (*Genomic Equivalence; ****p. < 0.0001*).

4. Establishment of iQC index for ddEGFR using FFPET samples

We classified all 316 samples into four groups according to their storage durations, DIN values, and iQC copies, as illustrated in Figure 4A. First, we analyzed DIN values for 57 discordant samples from among 169 samples derived from blocks stored for more than 6 years. The DIN value was less than 2.5 for most of the discordant samples (Fig. 15). Among 147 samples with a block storage duration ≤ 6 years, 26 samples did not satisfy our DIN criteria, and were included in Group 1 (Table 12, Table 13). To establish iQC index criteria, the 60 discordant samples in Group 1 were re-analyzed, and almost all (58/60) had an iQC index < 0.5 (Fig. 16).

Based on this result, the sample criteria were established as follows: block storage duration ≤ 6 years, DIN > 2.5, and iQC index ≥ 0.5. In addition, we observed a strong correlation between ddEGFR iQC index and DIN value, further supporting the idea that iQC index represents the quality of FFPET-DNA (*p. < 0.0001*, Table. 14).

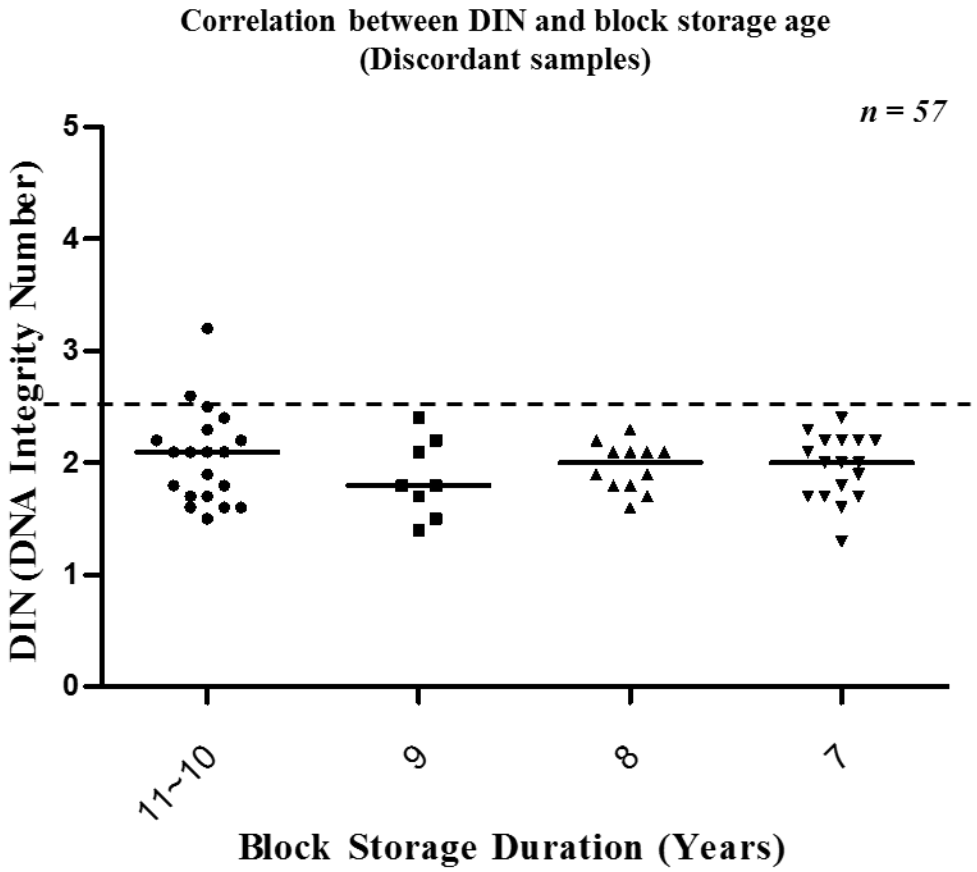


Figure 15. Establishment of sample criteria using discordant samples (DIN value). Discordant samples were analyzed by comparing the results of the Cobas EGFR and ddEGFR tests. Plots show distributions of the correlation between DIN value and sample storage duration of discordant samples. The black line represents the median value. The DIN values of discordant samples are distributed under the black dotted line.

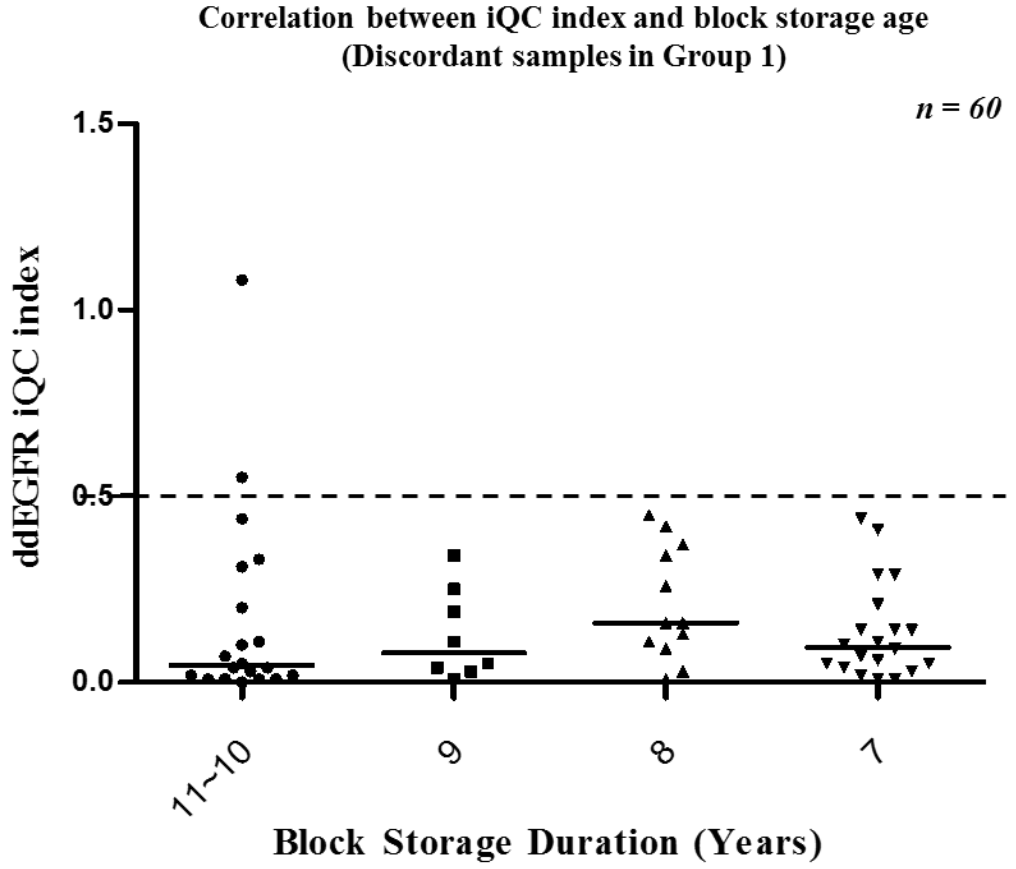


Figure 16. Establishment of sample criteria using discordant samples (iQC index). Discordant samples were analyzed by comparing the results of the Cobas EGFR and ddEGFR tests. Plots show distributions of the correlation between iQC index and sample storage duration of discordant samples. The black line represents the median value. The iQC index of discordant samples are distributed under the black dotted line.

Group 1 (*n = 194*)		cobas EGFR Test		
		MD	**MND**	**Total**
ddEGFR Test	**MD**	49	56 *	105
	MND	9	79	88
	Total	58	135	193
PPA (95% C.I.)	84.48% (72.58% to 92.65%)			
NPA (95% C.I.)	58.52% (49.73% to 66.93%)			
OPA (95% C.I.)	66.32% (59.18% to 72.95%)			
PPV (95% C.I.)	46.67% (36.87% to 56.66%)			
NPV (95% C.I.)	89.77% (81.47% to 95.22%)			

Table 12. Comparison of ddEGFR and cobas EGFR results from FFPE samples (block storage duration > 6, DIN < 2.5). PPA, NPA, and OPA between the ddEGFR and cobas EGFR tests for the detection of *EGFR* mutations were 84.48%, 58.52%, and 66.32%, respectively. MD, mutation detected; MND, mutation not detected.

Group 1 (*n = 194*)		Cobas EGFR Test														
		G719X	19del	T790M	E20Ins	S768I	L858R	G719X, T790M	G719X, S768I	19del, T790M	19del, L858R	T790M, L858R	T790M, E20Ins	S768I, L858R	N/A	Total
ddEGFR Mutation Test	G719X	0														0
	19del		33												3	36
	T790M			0												0
	E20Ins				0										1	1
	S768I					0										0
	L858R		1 †				14								44	59
	G719X,T790M							0							1	1
	G719X,S768I								1							1
	19del,T790M		3							0						3
	19del,L858R		1								0					1
	T790M,L858R											0			1	1
	T790M,E20Ins				1								0			1
	S768I, L858R					1								1		2
	N/A		9												79	88
	Total	0	47	0	1	1	14	0	1	0	0	0	0	1	129	194

κ coefficient = **0.4611** (95% C.I. 36.56−55.66%)

Table 13. Detail concordance rate of group 1 samples (block storage duration > 6, DIN < 2.5).

	iQC index / DIN	Bad (< 2.5)	Good (> 2.5)
Group 1 (*n = 194*) (*p. < 0.0001*)	**Low (< 0.5)**	155	2
	High (≥ 0.5)	22	15
Group 2 (*n = 113*)	**Low (< 0.5)**	0	8
	High (≥ 0.5)	0	113
Group 3 (*n = 150*)	**Low (< 0.5)**	0	0
	High (≥ 0.5)	22	128
Group 4 (*n = 166*)	**Low (< 0.5)**	156	10
	High (≥ 0.5)	0	0

Table 14. Correlation between ddEGFR iQC index and DIN value. The ddEGFR iQC index and DIN value of excluded samples were lower than the values of the sample criteria. Results from included samples were opposite to those of the excluded samples. In addition, the ddEGFR iQC index and DIN values were strongly correlated.

5. Comparison of ddEGFR and cobas EGFR tests with iQC index criteria

When the iQC index cut-off was applied to 121 samples (block storage duration ≤ 6 years, DIN > 2.5), 113 samples remained (Fig. 7, Group 2). Group 2 samples had a very high concordance rate between the ddEGFR and cobas EGFR tests (PPA = 100.00%, NPA = 76.00%, OPA = 94.69%, κ = 0.9197) (Table. 15, Table. 16). To determine the clinical implications of the iQC index, we applied this criterion to 316 FFPETs, resulting in the classification of 150 samples into Group 3 (Fig. 7), and re-analyzed the concordance rate between the ddEGFR and cobas EGFR results. Group 3 samples had a very high concordance rate (PPA = 100.00%, NPA = 75.00%, OPA = 92.67%, κ = 0.8923) (Table. 17, Table. 18), similar to the results from Group 2 (applying all criteria). By contrast, Group 4 samples, in which the iQC index criterion was not satisfied, exhibited a very low concordance rate (PPA = 78.57%, NPA = 58.20%, OPA = 63.41%, κ = 0.3862) (Table. 19, Table. 20). Therefore, we suggested that iQC index is a key factor in determining whether DNA is of sufficient quality for the ddEGFR test.

Group 2 (*n = 113*)		cobas EGFR Test		
		MD	**MND**	**Total**
ddEGFR Test	**MD**	88	6	94
	MND	0	19	19
	Total	88	25	**113**
PPA (95% C.I.)		100.0% (95.89−100.0%)		
NPA (95% C.I.)		76.00% (54.87−90.64%)		
OPA (95% C.I.)		94.69% (88.80−98.03%)		
PPV (95% C.I.)		93.62% (86.62−97.62%)		
NPV (95% C.I.)		100.0% (82.35−100.0%)		

MD, mutation detected; MND, mutation not detected

Table 15. Comparison of ddEGFR and cobas EGFR results from FFPE samples eliminated by the sample criteria (DIN and iQC index). Analysis of concordance rates between ddEGFR and cobas EGFR tests when the sample criteria were applied (Group 2). PPA, NPA, and OPA between the ddEGFR and cobas EGFR tests for the detection of *EGFR* mutations were 100%, 76.00%, and 94.69%, respectively. MD, mutation detected; MND, mutation not detected.

Group 2 (*n = 113*)		cobas EGFR Test										
		G719X	**19del**	**T790M**	**E20Ins**	**S768I**	**L858R**	**G719X, T790M**	**G719X, S768I**	**S768I, L858R**	**MND**	**Total**
ddEGFR Test	**G719X**	1									1	2
	19del		51								1	52
	T790M			0								0
	E20Ins				0						1	1
	S768I					0						0
	L858R						33				3	36
	G719X, T790M							0				0
	G719X, S768I								2			2
	S768I, L858R									1		1
	MND										19	19
	Total	1	51	0	0	0	33	0	2	1	25	113

κ coefficient = **0.9197** (95% C.I. 85.85−98.09%)

Table 16. Comparison between the ddEGFR and cobas EGFR test results with application of the sample criteria (Group 2).

Group 3 (*n = 150*)		cobas EGFR Test		
		MD	**MND**	**Total**
ddEGFR Test	**MD**	106	11 *	117
	MND	0	33	33
	Total	106	44	150
PPA (95% C.I.)	100.0% (96.58−100.0%)			
NPA (95% C.I.)	75.00% (59.66−86.81%)			
OPA (95% C.I.)	92.67% (87.26−96.28%)			
PPV (95% C.I.)	90.60% (83.80−95.21%)			
NPV (95% C.I.)	100.0% (87.26−100.0%)			

* 3 samples: cobas, 19del; ddEGFR, 19del/T790M

Table 17. Comparison of ddEGFR and cobas EGFR results from FFPE samples eliminated by the sample criteria (iQC index only). Analysis of concordance rates between ddEGFR and cobas EGFR tests when the sample criteria were applied (Group 3). PPA, NPA, and OPA between the ddEGFR and cobas EGFR tests for the detection of *EGFR* mutations were 100%, 75%, and 92.67%, respectively. MD, mutation detected; MND, mutation not detected.

Group 3 (*n = 150*)		cobas EGFR Test											
		G719X	**19del**	**T790M**	**E20Ins**	**S768I**	**L858R**	**G719X, T790M**	**G719X, S768I**	**19del,T790M**	**S768I, L858R**	**MND**	**Total**
ddEGFR Test	**G719X**	1										1	2
	19del		61									1	62
	T790M			0									0
	E20Ins				0							1	1
	S768I					0							0
	L858R						41					4	45
	G719X,T790M							0				1	1
	G719X,S768I								2				2
	19del,T790M		3							0			3
	S768I, L858R										1		1
	MND											33	33
	Total	1	64	0	0	0	41	0	2	0	1	41	150

κ coefficient = **0.8923** (95% C.I. 83.25−95.22%)

Table 18. Comparison between the ddEGFR and cobas EGFR test results, with application of only the iQC criterion (Group 3).

Group 4 (*n = 166*)		cobas EGFR Test		
		MD	**MND**	**Total**
ddEGFR Test	**MD**	33	51 *	84
	MND	9	71	80
	Total	42	122	164
PPA (95% C.I.)	78.57% (63.19−89.70%)			
NPA (95% C.I.)	58.20% (48.93−67.06%)			
OPA (95% C.I.)	63.41% (55.55−70.79%)			
PPV (95% C.I.)	39.29% (28.80−50.55%)			
NPV (95% C.I.)	88.75% (79.72−94.72%)			

* 1 sample: cobas, 19del; ddEGFR, 19del/L858R

1 sample: cobas, E20Ins; ddEGFR, E20Ins/T790M

1 sample: cobas, S768I; ddEGFR, S768I/L858R

Table 19. Re-analysis of concordance rate using excluded samples. Samples were selected by iQC index (< 0.5). The 166 excluded samples with valid ddEGFR test and cobas EGFR test results were included in the agreement analysis. PPA, NPA, and OPA between the ddEGFR and cobas EGFR tests for the detection of *EGFR* mutations were 78.57%, 58.20%, and 63.41%, respectively.

Group 4 (*n = 166*)		cobas EGFR Test														
		G719X	**19del**	**T790M**	**E20Ins**	**S768I**	**L858R**	**G719X, T790M**	**G719X, S768I**	**19del, T790M**	**19del, L858R**	**T790M, E20Ins**	**T790M, L858R**	**S768I, L858R**	**N/A**	**Total**
ddEGFR Test	**G719X**	0														0
	19del		24												3	27
	T790M			0												0
	E20Ins				0										1	1
	S768I					0										0
	L858R		1 †				7								43	51
	G719X, T790M							0								0
	G719X, S768I								1							1
	19del,T790M									0						0
	19del,L858R		1								0					1
	T790M,E20Ins				1							0				1
	T790M,L858R												0		1	1
	S768I, L858R					1								1		2
	N/A		9												71	80
	Total	0	35	0	1	1	7	0	1	0	0	0	0	1	119	165

† 1 sample was excluded from the analysis, 1 sample: invalid

κ coefficient = 0.3862 (95% C.I. 27.85−49.40%)

Table 20. Detail concordance rate using excluded samples. Samples were selected by iQC index (< 0.5). The 166 excluded samples with valid ddEGFR test and cobas EGFR test results were included in the agreement analysis.

6. Analysis of discordant samples in pre-clinical study

Applying the iQC index criterion, we re-analyzed the remaining 11 discordant samples in Group 3. A schematic representation of the re-analysis workflow for discordant samples is depicted in Figure 17. In three of the samples, the ddEGFR test reported a double mutation (19del/T790M), whereas the cobas EGFR test and Sanger sequencing reported only a single mutation (19del) (Table 17, Group 3). This may be a result of the low detection sensitivity of Sanger sequencing (around 15%) (128) and cobas EGFR tests (LoD of T790M = ~ 3%; cobas EGFR v2). Based on the ddEGFR results of these three samples, the MI of T790M was ~1% (1.11%, 1.16%, and 1.03%). Additionally, eight discordant samples were verified by Sanger sequencing, and no mutations were found (Table. 21). Moreover, to observe the effect of tumor ratio, we performed macrodissection to enrich for tumor tissue, and then re-analyzed *EGFR* mutations in eight samples for which the cobas EGFR test had yielded negative results but the ddEGFR test yielded positive results. After macrodissection, the cobas EGFR test gave the same results as the ddEGFR test for four of the eight samples (Table. 21). Thus, our results indicate that the ddEGFR test is more sensitive for EGFR mutation detection, independent of tumor ratio. Strangely, one discordant case was a mutation detected (T790M/G719X) of preliminary result, whereas invalid by the ddEGFR test after macrodissection. Because iQC index is very low (0.37, data not shown), it is estimated that DNA degradation progressed during the macrodissection process.

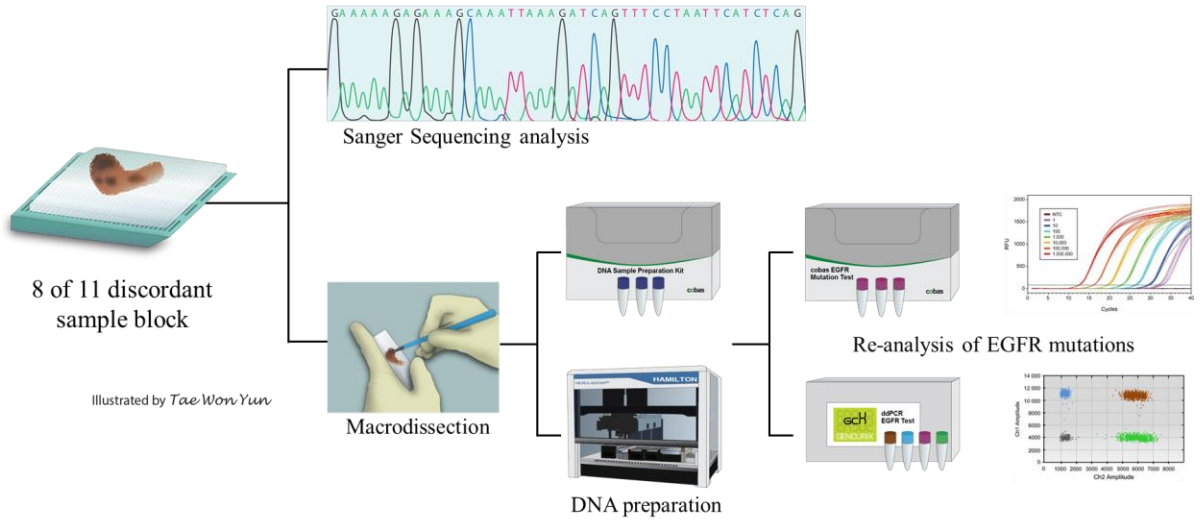


Figure 17. Schematic of re-analysis of discordant samples. Eight of eleven discordant samples were verified by Sanger sequencing. In addition, after increasing the tumor-to-normal tissue ratio, *EGFR* mutations were re-analyzed using the cobas EGFR and ddEGFR tests.

				Preliminary result						After Macrodissection				
Sample No	**Block Storage age**	**C/N Ratio (%)**	**DIN**	**cobas**		**ddEGFR**		**ddEGFR MI(%)**	**Sanger**	**cobas**		**ddEGFR**		**ddEGFR MI (%)**
1	11	23	3.2	MND	-	MD	L858R	0.8	WT	N/A	N/A	N/A	N/A	-
2	11	51	2.2	MND	-	MD	T790M/G719X	1.08 / 1.02	Invalid	MND	-	Invalid	-	-
3	6	41	4.6	MND	-	MD	L858R	1.57	WT	MD	L858R	MD	L858R	2.57
4	6	28	3.7	MND	-	MD	G719X	10.17	WT	MD	G719X	MD	G719X	20.17
5	6	12	3.9	MND	-	MD	L858R	5.91	WT	MD	L858R	MD	L858R	4.55
6	6	15	3.1	MND	-	MD	L858R	7.4	WT	N/A	N/A	N/A	N/A	-
7	3	43	4.4	MND	-	MD	20Ins	12.88	WT	MND	-	MD	E20Ins	7.4
8	3	34	4.1	MND	-	MD	19del	15.72	WT	MD	19del	MD	19del	3.15

MD, mutation detected; MND, mutation not detected.

N/A, FFPE blocks not available

Table 21. Re-analysis of eight of eleven discordant samples in Group 3. Eight of eleven discordant samples were verified by Sanger sequencing. In addition, after increasing the tumor-to-normal tissue ratio, detection of EGFR mutations was re-analyzed using the cobas EGFR and ddEGFR tests. After enrichment of tumor tissue, the cobas EGFR test could identify previously undetected *EGFR* mutations in four out of the eight samples. However, the ddEGFR analysis yielded data identical to the preliminary results.

7. Retrospective comparative clinical study for clinical utility of iQC index

Next, we analyzed the *EGFR* mutation status of 228 samples using the ddEGFR and cobas EGFR tests; 57 samples were excluded based on the iQC index (Fig. 8). The remaining 171 samples with iQC index ≥ 0.5 gave PPA of 98.23%, NPA of 82.76%, and OPA of 92.98% between the ddEGFR and cobas EGFR tests (κ = 0.9029, Table. 22, Table. 23). Among 12 discordant samples, six were reported to have a double mutation according to the ddEGFR test but only a single mutation according to the cobas EGFR test. As expected, the MI of the additional detected mutation was very low. One discordant case was a mutation not detected by the cobas EGFR test, but detected (L861Q) by both ddEGFR test and Sanger. Conversely, another discordant case was a mutation not detected by the ddEGFR test but detected (19del) by the cobas EGFR test and Sanger (Table. 24). This was a rare mutation of the 19del subtype (c.2239_2264del_insGCGAA) caused by a non-specific reaction that is not designed in the cobas EGFR test, and thus it cannot be employed to discriminate the possibility potential erroneous detection (129) and beneficial cross-reaction of commercial diagnostic kits.

Retrospective comparison study, Applied iQC index (*n = 171*)		cobas EGFR Test		
		MD	**MND**	**Total**
ddEGFR Test	**MD**	111	10 *	121
	MND	2	48	50
	Total	113	58	171
PPA (95% C.I.)		98.23% (93.75−99.78%)		
NPA (95% C.I.)		82.76% (70.57−91.41%)		
OPA (95% C.I.)		92.98% (88.06−96.32%)		
PPV (95% C.I.)		91.74% (85.33−95.97%)		
NPV (95% C.I.)		96.00% (86.29−99.51%)		

* 3 samples: Cobas, 19del; ddEGFR, 19del/T790M

1 sample: Cobas, G719X; ddEGFR, G719X/L861Q

1 sample: Cobas, L858R; ddEGFR, G719X/L858R

1 sample: Cobas, L858R; ddEGFR, T790M/L858R

Table 22. Method correlation between ddEGFR and cobas EGFR test in Retrospective comparison study group. Samples with valid ddEGFR and cobas EGFR test results were included in the agreement analysis. PPA, NPA, and OPA between the ddEGFR and cobas EGFR tests for the detection of *EGFR* mutations were 98.23%, 82.76%, and 92.98%, respectively. MD, mutation detected; MND, mutation not detected.

Retrospective comparison study, Applied iQC index (*n = 171*)		cobas EGFR Test													
		G719X	**19del**	**T790M**	**E20Ins**	**S768I**	**L858R**	**L861Q**	**19del, T790M**	**G719X, S768I**	**G719X, L858R**	**G719X, L861Q**	**T790M, L858R**	**N/A**	**Total**
ddEGFR Test	**G719X**	2													2
	19del		46												46
	T790M			0											0
	E20Ins				2									1	3
	S768I					0									0
	L858R						54							2	56
	L861Q							0						1	1
	19del,T790M		3						1						4
	G719X,S768I									3					3
	G719X,L858R						1				0				1
	G719X,L861Q	1										0			1
	T790M,L858R						1						3		4
	N/A		2											48	50
	Total	3	51	0	2	0	56	0	1	3	0	0	3	52	171

κ coefficient = **0.9029** (95% C.I. 85.08−95.49%)

Table 23. Detailed concordance rate of the retrospective comparison study group.

Sample No.	cobas EGFR test		ddEGFR test		ddEGFR MI		Sanger Seq
1	MND	-	MD	L858R	L858R, 25.47%		Invalid
2	MD	L858R	MD	L858R/T790M	L858R, 42.9%	T790M, 1.01%	L858R
3	MD	19del	MD	19del/T790M	19del, 12.79%	T790M, 0.96%	19del
4	MD	L858R	MD	L858R/G719X	L858R, 43.22%	G719X, 1.98%	Invalid
5	MD	19del	MD	19del/T790M	19del, 22.45%	T790M, 1.22%	19del
6	MD	19del	MD	19del/T790M	19del, 47.94%	T790M, 0.87%	19del
7	MD	19del	MND	-	-		WT
8	MND	-	MD	E20Ins	E20Ins, 14.55%		Invalid
9	MD	19del	MND	-	-		19del *
10	MND	-	MD	L861Q	L861Q, 64.93%		L861Q
11	MD	G719X	MD	G719X/L861Q	G719X, 11.36%	L861Q, 13.64%	Invalid
12	MND	-	MD	L858R	L858R, 6.46%		Invalid

* This was a rare mutation of the 19del subtype (c.2239_2264del_insGCGAA) caused by a non-specific reaction that is not designed in the cobas EGFR test, and thus it cannot be employed to discriminate the possibility potential erroneous detection (129).

Table 24. Discordant analysis by Sanger sequencing in the retrospective comparison study group. Based on the results of the retrospective comparison with cobas EGFR test, 12 samples were valid and discordant with the ddEGFR test. Samples that gave discordant results between the cobas EGFR and ddEGFR tests were analyzed by Sanger sequencing. MD, mutation detected; MND, mutation not detected.

In addition, we measured DIN values from 228 FFPET-DNA samples and observed a pattern similar to that of iQC index. Furthermore, the majority of the most recent samples (within 1 year) had DIN > 2.5 and iQC index ≥ 0.5 (Fig. 18). These data revealed that iQC index is a very powerful indicator of the quality of FFPET-DNA. In addition, these observations demonstrate that the ddEGFR test is a robust diagnostic tool for the accurate detection of *EGFR* mutations in clinical practice.

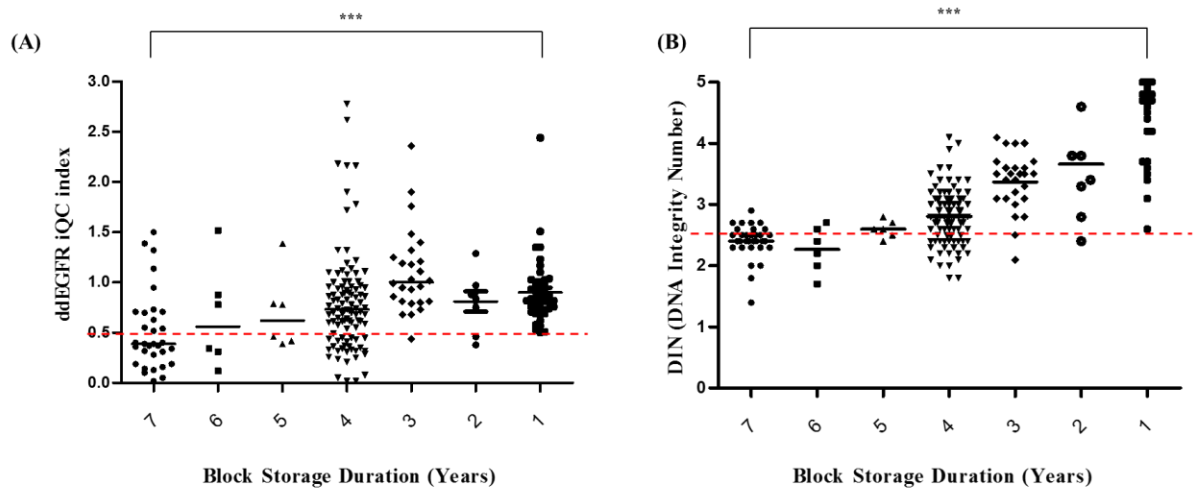


Figure 18. Distributions of iQC index and DIN in the retrospective comparative study group. Most of the more recent samples (taken within 1 year) satisfied the sample criteria defined above (red dotted line) (***$p. < 0.0001$).

V. Discussion

FFPETs undergo effective preservation of the cellular, architectural, and morphological details and allow easy storage at room temperature for extensive periods. For these reasons, FFPET is useful source for molecular diagnostics. But, the quality of FFPET-DNA has been largely ignored in the clinical research and diagnosis field, and internal control of most commercial diagnostic kits were used only to validate assays. Changes in mutation status due to the low quality of FFPET-DNA may result in incorrect diagnoses. Therefore, considerable effort is required to optimize the sample criteria for determining the quality of FFPET-DNA that is suitable for PCR. In this study, we established iQC index criteria to determine the minimum quality of FFPET-DNA and demonstrated the benefits of implementing these criteria benefits in a real-world clinical application.

In our experiments, both automated tissue preparation system (TPS; Siemens Healthcare, Erlangen, Germany), which can minimize handling errors and decrease the effect of formaldehyde-induced DNA–DNA and DNA–protein crosslinks (51, 58), and UDG treatment are powerful strategy for reducing false positives caused by sequence artifacts (61, 130-132). However, sequence artifacts due to DNA fragmentation remained a problem. Limitations in the availability of many clinical specimens drive the need for low DNA inputs into molecular assays. Cutting edge technologies such as NGS required good quality of DNA material for in-depth molecular profiling in various cancer (133). None of traditional tools such as UV absorbance, gel electrophoresis, assays with intercalating dyes measure the amount of amplifiable DNA, and each of these tools has an additional limitations (134). Also, depending on the degree of fragmentation, the same quantities of DNA from different FFPET samples can contain widely different amounts of amplifiable DNA templates (59). For this reason, PCR-based methods such as qPCR, ddPCR, and next-generation sequencing (NGS) are preferable for quantifying the amount of amplifiable template in FFPET-DNA (119).

iQC copies can represent the concentration of input DNA (Fig. 11, 12) and a novel concept for mutation calling, MI, was introduced as an indicator of the mutation level, which in turn reflects DNA quality. MI provides a much more accurate indication of the mutation level than mutation frequency, which is simply calculated based on input DNA concentration. Accordingly, we designed an iQC that measures *EGFR* exon 20 to assess the amount of amplifiable FFPET-DNA. Because elevated *EGFR* expression have been observed in NSCLC (128), the internal control should be designed to represent the levels of the *EGFR* gene copy number.

Interestingly, some discordant samples reported as single mutations by the cobas EGFR test were shown to have double mutation by the ddEGFR test in pre-clinical (Table. 17) and clinical studies (Table. 22). The additional detected MI was around 1%, as expected. According to a clonal selection model, EGFR-TKI treatment may lead to the selection of T790M mutant cells, and thus even a small fraction of T790M positive tumor cells at the beginning of treatment could lead to clinical EGFR-TKI resistance (25). Therefore, is very important to detect even a small fraction of mutations.

The discordant results were largely due to the difference in LoD between both tests. Specifically, some mutations detected by the ddEGFR test cannot be detected by the cobas EGFR test because the sensitivity of the ddEGFR test is higher. In the pre-clinical study Group 3, L858R MIs were relatively low in cobas EGFR-negative samples (around 5%, Table. 21). Considering that the LoD of the cobas EGFR test was 5%, these MI values, which reflect sample quality and mutation level, are reasonable. When the iQC index was applied, the proportion of such discordant samples was small.

The cobas EGFR test recommends macrodissection of low-percent tumor tissues (below

10%) to improve detectability (126). When the cobas EGFR test yielded a result of 'mutation not detected' (MND), even though the C/N ratio was over 10%, the *EGFR* mutation was re-analyzed after increasing the tumor ratio by macrodissection. The cobas EGFR test yielded the same results as the ddEGFR test for four of the eight samples (Table. 21), showing that ddEGFR exhibits superior analytical performance. Thus, the detectability of the cobas EGFR test may be increased by enriching for tumor tissue, e.g., by macrodissection, but this manipulation requires extra time and effort. By contrast, the ddEGFR test does not require macrodissection and improves reliability.

For 1-year-old samples (*n = 46*) in the clinical study group, all iQC index and DIN values satisfied the sample criteria (iQC index ≥ 0.5, DIN > 2.5) (Fig. 18), suggesting that the low age samples used in clinical practice will have minimal problems with DNA quality. Previous reported that, cobas EGFR test and Sanger are highly concordant because used to low age samples (50, 135, 136). In contrast, concordance rate between cobas EGFR test and Sanger of the pre-clinical study samples were very low (Table. 10, Table. 11), but when iQC index was applied, the concordance rate was increased (Data not shown). Given the additional cost and effort required for DIN measurement, the iQC index is a robust indicator of the minimum DNA quality required for PCR amplification, as it takes into account the various types of DNA damage caused by FFPET processing and long-term storage.

In addition, iQC index ≥ 0.5 is an indicator that guarantees clinical equivalence obtained through comparative clinical studies with existing approved diagnostics test (cobas EGFR test). Clinical equivalence cannot be guaranteed for samples with an iQC index < 0.5. However, discordance of samples with iQC index < 0.5 can be explained by two causes. The first cause may be due to the possibility of false negative results of cobas EGFR test. Second, there is a possibility of false positive result due to ultra-sensitivity of ddEGFR test. The analytical

performance of the ddEGFR test indicates a high possibility of false negative results on the cobas EGFR test. However, it is questionable whether mutations detected in samples with an iQC index < 0.5 (especially mutations detected at low MI %) are related to drug treatment response. This must be verified through prospective clinical trials. Small-scale independent retrospective clinical studies for the ddEGFR test have shown responses of patients treated with an EGFR-TKI, even of patients with an iQC index < 0.5 (unpublished). Therefore, an iQC index ≥ 0.5 is a numerical value used in comparative clinical studies to guarantee clinical equivalence between the results of the ddEGFR and cobas EGFR tests. This means that iQC index may be changed through an independent prospective clinical trial for ddEGFR test. Also, using another approved kit for comparative clinical study, the iQC index may be changed for clinical equivalence. This is the result of considering clinical cut-off not only to compare the mutation results but also to ensure EGFR-TKI response.

We also observed that the iQC index could be used to obtain a diagnosis using other cancer type samples (colorectal cancer) and other diagnostic kits, including the ddPCR-based KRAS mutation test and the cobas® KRAS mutation test. Similar to the results of this study, we observed that when the iQC index was applied, the concordance rate between the results of the two kits increased significantly (data not shown). Overall, the iQC index could be a useful criterion for judging the quality of FFPET-DNA.

The ddEGFR test is designed to detect the C797S mutation in *EGFR* exon 20. NSCLC patients with advanced disease are usually treated with first or second generation EGFR-TKIs (erlotinib, gefitinib, or afatinib) (137, 138). However, patients acquire resistance after 9 to 13 months (34, 139, 140), the most common being T790M within the *EGFR* kinase domain (22, 24). Third generation EGFR-TKI have been designed to mutant-selectively target highly active against T790M mutation (141, 142). Recently, ctDNA evaluation was used to identify a novel

mechanism underlying acquired resistance to the third generation EGFR-TKI osimertinib; specifically, emergence of an acquired *EGFR* C797S mutation (33). The pre-clinical and retrospective clinical studies based on the ddEGFR test did not detect the C797S mutation. However, we subsequently found out that none of the NSCLC patients in those studies actually received a third generation EGFR-TKI.

Diagnostic kits must be able to detect mutations in very small amounts of DNA with high sensitivity. One of the problems associated with detecting *EGFR* mutations in NSCLC patients is not obtaining a sufficient quantity of specimen to confirm the status of several biomarkers. The ddEGFR test can detect *EGFR* mutations using only 3.3 ng (total 13.2 ng/4 wells) of FFPET-DNA per well, with a sensitivity of 0.78%. With cobas EGFR test, 3 reactions were performed using 50 ng DNA per well (total 150 ng / 3 well) to detect 42 *EGFR* mutations.

The presence of EGFR mutations in NSCLC is a major prognostic biomarker in terms of treatment selection. Thus, sensitive and accurate screening for EGFR mutations facilitates a personalized and specific approach to patient treatment / management.

In conclusion, detecting genetic mutations in FFPET samples is difficult because the DNA often becomes fragmented during storage, resulting in sequence artifacts and low-quality data. The iQC index allows selection of appropriate FFPET-DNA samples for companion diagnostics using a ddPCR-based mutation test. Furthermore, we suggest that clinical trials using FFPET should involve criteria that reflect the quality of the DNA in samples. The performance of the ddPCR-based EGFR mutation test developed herein was superior to that of the commercially available Cobas EGFR test.

VI. Conclusion and perspectives

Personalized treatment for cancer patients should include a genetic assessment of mutation status. FFPET is the most widely available material for molecular diagnostics and research. FFPET samples are routinely used for histopathological analysis because they are convenient, cost-effective, and easy to store. However, detecting genetic mutations in FFPET samples is difficult because DNA fragments during storage, resulting in sequence artifacts. DNA fragmentation is an irreversible reaction that not only reduces the amount of amplifiable template but also leads to stochastic enrichment of artifactual changes. However, since mutational analyses of FFPETs is a standard procedure used to inform therapeutic decisions, appropriate measures must be incorporated to allow assessment of DNA damage and prevent the calling of false-positive mutations.

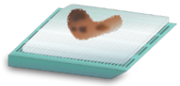
For reliable analysis of mutations, it is necessary to pre-analyze and evaluate the amount/quality of amplifiable template FFPE DNA. The more sensitive test method, the higher purity DNA is required. Traditional quantitation methods such as UV absorbance and spectrophotometry lack accuracy when it comes to measuring the amount of amplifiable template DNA. Evaluating the amount of amplifiable DNA is very important in diagnosis using PCR-based methods. Up to this time, the PCR-based diagnostic test reflected only the validity and invalidity of the assay, not considering the quality of the DNA. The iQC index established herein enables accurate detection of *EGFR* mutations. If using PCR-based assays to detect *EGFR* mutations, we believe that the results can be trusted if at least half of the input DNA is amplifiable. The iQC index allows appropriate selection of FFPET DNA samples for companion diagnosis using a ddPCR-based mutation test. In addition, DNA quality and EGFR mutations are analyzed simultaneously in same NSCLC FFPET sample. The results are reliable, even though only a small amount of FFPET DNA is required. Indeed, we found that the analytical performance of the ddPCR-based EGFR mutation test was superior to that of the

Cobas EGFR test. Measuring the quality of DNA extracted from patient samples makes it possible to prevent diagnostic errors, allow re-testing where appropriate, and make decisions regarding appropriate treatment (Fig. 19). The results of this study will also be of benefit to clinical researchers. As mentioned above, handling and storage procedures vary from site to site, and multi-center clinical trials that do not take sample quality into account can yield unreliable results; both of these problems can be overcomed by application of the iQC index and standardization of sample quality (Fig. 20).

The iQC index seems to be most useful for diagnoses based on liquid biopsy samples (e.g., ctDNA). The iQC index may be necessary for liquid biopsy specimens since the quality of ctDNA can vary diversely by frequent fragmentation, which is a biological property of ctDNA, variations in sample collection and storage by each hospital, and by logistic issues.

We are currently conducting prospective clinical studies to confirm the clinical utility of the ddPCR-based EGFR mutation test for liquid biopsy samples. A ddEGFR test based on plasma samples may be both feasible and effective for identifying EGFR mutations, predicting treatment responses, and monitoring acquired resistance during EGFR-TKI treatment. T790M is the most common mutation associated with acquired resistance to first-generation EGFR-TKIs (23). T790M is present in a small population of tumor cells during development of NSCLC; therefore, it may be detected at very low frequency (25). A very low level T790M not detected by Cobas EGFR can be sensitively detected with ddEGFR test. However, it is very difficult to verify using the gold standard method such as Sanger sequencing due to technical limitations (low sensitivity). Therefore, we intend to undertake a prospective clinical trial to examine the usefulness of the ddEGFR test for monitoring the activity of EGFR-TKIs in NSCLC patients harboring T790M at low frequency; this will inform us as to whether the test can predict patients that will benefit from treatment with third generation EGFR-TKIs.

In the near future, mutation detection tests will play a very important role in the clinical setting as they will identify biomarkers for molecular targeted therapy. The benefits of treatment will be maximized only if accurate companion diagnostics are used; these diagnostics must be validated according to strict standards. The ddEGFR test not only detects *EGFR* mutations but also provides information about the quality of DNA, thereby preventing diagnostics errors. It also offers the opportunity to decide when to appropriate treatment through serial monitoring using liquid biopsy (Fig. 21).



ex> Input DNA 1000 copies

Ex> Input DNA 1000 copies			ddEGFR test			Cobas EGFR test	
Patient No.	Amplifiable DNA copies	Mut DNA copies	**iQC index** (amplifiable DNA copy/ Input DNA copies)	**MI %** (Mut copy/Amplifiable DNA copy) x 100	**Result**	**Mutation freq %** (Mut copy/Input DNA copy) x 100	**Result**
Patient 1	**1000**	**100**	1000/1000 = 1	100/1000 x 100 = 10	MD	100/1000 x 100 = 10	MD
Patient 2	**500**	**50**	500/1000 = 0.5	50/500 x 100 = 10	MD	50/1000 x 100 = 5.0	MD
Patient 3	**250**	**25**	250/1000 = 0.25	25/250 x 100 = 10	Invalid	25/1000 x 100 = 2.5	MND

MD: Mutation detected
MND: Mutation not detected

Figure 19. Clinical meaning of iQC index (risk management). Prevent diagnostic errors due to DNA quality and risk management is possible.

Figure 20. Clinical meaning of iQC index (viewpoint of multi-center clinical and translational research). Since conditions for processing and storage of samples from each clinical institution are different, it is necessary to provide an indicator for the quality of DNA.

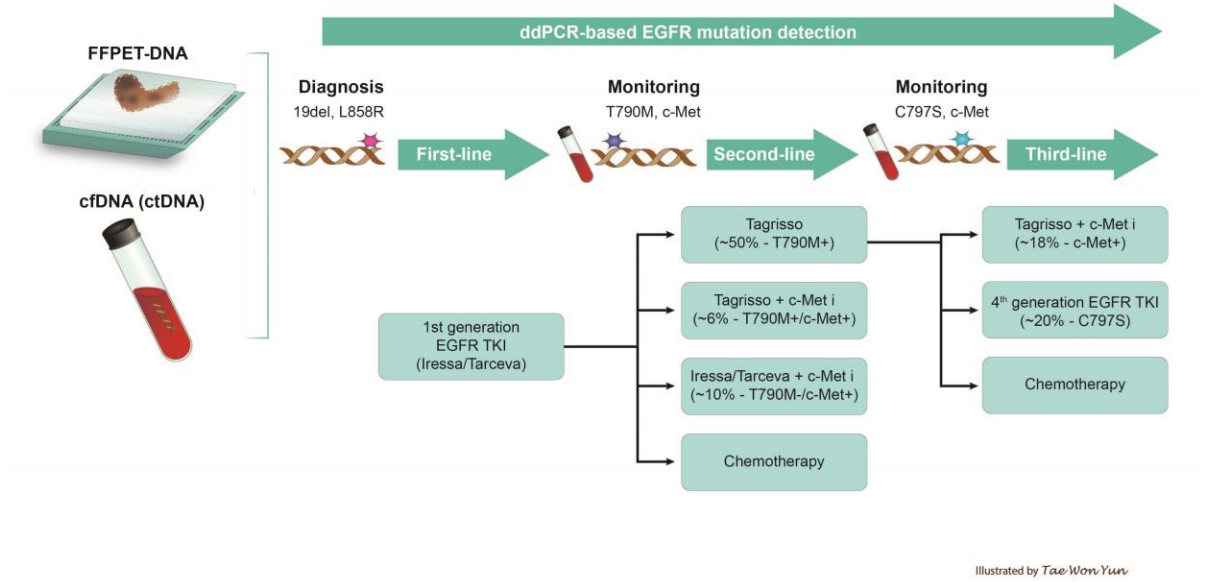


Figure 21. Dynamic monitoring of ctDNA in NSCLC. ddPCR-based mutation test can be used for the quantification of dynamic changes in the *EGFR, c-Met* mutations in ctDNA, and can be used to assess the clinical outcomes of therapy.

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국문 초록

DNA의 특질 분석을 이용한 드롭렛 디지털 PCR 기반의 *EGFR* 돌연변이 검출 방법 연구

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포르말린 고정 파라핀 내장 조직 (formalin-fixed paraffin-embedded tissue, FFPET) 샘플은 임상 연구와 체외 진단분야에 있어서 매우 중요한 자원이다. 하지만, 포르말린 고정 파라핀 내장 조직 샘플을 이용한 유전자 돌연변이 검출은 포르말린 고정 파라핀 내장 조직의 처리 과정 및 다양한 보존기간과 품질로부터 기인하는 문제점으로 인하여 부정확한 결과를 초래한다.

본 연구는 315 개의 비소세포 폐암 환자의 FFPET-DNA 샘플을 사용한 전임상 연구를 통하여 ddPCR 기반 (GenesWell™ ddEGFR Mutation test, “ddEGFR test”)과 Real-time PCR 기반의 EGFR 돌연변이 테스트 (cobas® EGFR Mutation test, “cobas EGFR test”) 를 비교함으로써 PCR에 적합한 최소 DNA 품질을 반영하는 샘플 기준을 확립했다. 확립된 샘플 기준을 사용하여 171개의 비소세포 폐암 환자의

FFPET-DNA 샘플을 사용한 독립된 후향적 임상 연구에서 ddEGFR 및 cobas EGFR test에 대한 비교 임상 및 샘플 기준의 임상적 유용성을 평가 하였다.

전임상 연구를 통하여 FFPET-DNA의 샘플 기준 (iQC index ≥ 0.5, iQC copies 500 이상, FFPET-DNA가 3.3 ng [1000 GE]을 사용)을 확립 하였고, 이는 Input DNA 농도를 기준으로 절반 이상이 증폭 가능함을 의미한다. iQC index를 적용한 전임상 샘플 150개를 재 분석 하였고, ddEGFR test와 Cobas EGFR test의 일치율이 92.67%로 증가 하였다 (전임상 샘플 315개 전체 일치율 = 78.10%). iQC index를 기반으로, 독립적인 후향적 임상 시험을 실시 하였고, ddEGFR test와 Cobas EGFR test의 일치율이 매우 높게 관찰 되었다 (OPA = 92.98%). 또한, cobas EGFR test에서 검출하지 못하는 샘플들을 ddEGFR test를 사용하여 민감하게 검출 하였다.

iQC index는 FFPET-DNA 의 품질을 반영하는 신뢰할 수 있는 지표이며 매우 낮은 품질의 샘플로부터 기인하는 부정확한 진단을 방지할 수 있다. 또한, 기허가 받은 cobas EGFR test와 비교하여, ddEGFR test는 우수한 분석적 성능 및 적어도 동등한 임상적 성능을 나타냈다.

주요어: 상피세포성장수용체; 포르말린 고정 파라핀 내장 조직; 핵산 무결성 지수; 드롭렛 디지털 중합효소 연쇄반응; 유전자 돌연변이 검출; 핵산 내부 품질 지수

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