Review Article
Diagnostic Genetics

Ann Lab Med 2022;42:141-149
https://doi.org/10.3343/alm.2022.42.2.141
ISSN 2234-3806 • eISSN 2234-3814

ANNALS OF LABORATORY MEDICINE

Clinical Practice Guidelines for Pre-Analytical Procedures of Plasma Epidermal Growth Factor Receptor Variant Testing

Saeam Shin, M.D., Ph.D.[1,*], Hye In Woo, M.D., Ph.D.[2,*], Jong-Won Kim, M.D., Ph.D.[2], Yoonjung Kim M.D., Ph.D.[1], Kyung-A Lee, M.D., Ph.D.[1]

[1]Department of Laboratory Medicine, Yonsei University College of Medicine, Seoul, Korea; [2]Department of Laboratory Medicine and Genetics, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea

Standardization of cell-free DNA (cfDNA) testing processes is necessary to obtain clinically reliable results. The pre-analytical phase of cfDNA testing greatly influences the results because of the low proportion and stability of circulating tumor DNA (ctDNA). In this review, we provide evidence-based clinical practice guidelines for pre-analytical phase procedures of plasma epidermal growth factor receptor gene (*EGFR*) variant testing. Specific recommendations for pre-analytical procedures were proposed based on evidence from the literature and our experimental data. Standardization of pre-analytical procedures can improve the analytical performance of cfDNA testing.

Key Words: Cell-free nucleic acid, Circulating tumor DNA, Pre-analytical phase, Epidermal growth factor receptor, Clinical practice guidelines

Received: December 31, 2020
Revision received: February 2, 2021
Accepted: July 27, 2021

Corresponding author:
Kyung-A Lee, M.D., Ph.D.
Department of Laboratory Medicine, Yonsei University College of Medicine,
211 Eonju-ro, Gangnam-gu, Seoul 06273, Korea
Tel: +82 2-2019-3531
Fax: +82 2-2019-4822
E-mail: KAL1119@yuhs.ac

Co-corresponding author:
Yoonjung Kim, M.D., Ph.D.
Department of Laboratory Medicine, Yonsei University College of Medicine,
211 Eonju-ro, Gangnam-gu, Seoul 06273, Korea
Tel: +82-2-2019-3775
Fax: +82-2-2019-4822
E-mail: WSYJ1101@yuhs.ac

*These authors contributed equally to this work.

INTRODUCTION

Epidermal growth factor receptor gene (*EGFR*) variants are predictive markers of EGFR-tyrosine kinase inhibitor (TKI) responses in patients with non-small cell lung cancer (NSCLC) [1]. For many NSCLC patients, tumor tissue samples are not available for *EGFR* variant testing. Liquid biopsy using tumor-derived cell-free DNA (cfDNA) in patient blood is increasingly used for

the molecular characterization of tumors [2]. Plasma *EGFR* variant testing for circulating tumor DNA (ctDNA) has been performed in Korea since 2017 to select patients in whom EGFR-TKIs may be effective [3]. Currently, in Korea, several platforms are available for clinical *EGFR* variant testing using ctDNA, such as Cobas EGFR Mutation Test v2 (Roche Molecular Systems, Basel, Switzerland), PANAMutyper R EGFR (Panagene, Daejeon, Korea), TheraScreen EGFR RGQ PCR kit (Qiagen, Hilden, Germany), and droplet digital PCR. According to the test sensitivity, the concordance rate between plasma and tissue results for detecting p.T790M variant varies from 48% to 94% [4, 5].

In addition to the differences in test platforms, the pre-analytical phase of cfDNA testing largely influences the test results [6]. Optimization of ctDNA testing is challenging because of the very low proportion of ctDNA in the background wild-type cfDNA and a high susceptibility of short-fragmented cfDNA to degradation [6, 7].

To achieve optimal performance of plasma *EGFR* variant testing in clinical laboratories, standard guidelines for the pre-analytical phase of the test process are necessary. We developed evidence-based clinical practice guidelines for pre-analytical procedures for plasma *EGFR* variant testing. To suggest points of improvement in the current state of testing, we surveyed the actual practice of plasma *EGFR* variant testing in Korean clinical laboratories.

SURVEY ON CURRENT LABORATORY PRACTICE

The survey was conducted between December 2018 and January 2019 in 19 Korean clinical laboratories performing or referring for plasma *EGFR* variant testing. The laboratory directors volunteered to complete a self-administered anonymous questionnaire via e-mail regarding pre-analytical and analytical laboratory strategies for plasma *EGFR* variant testing.

The results of this survey are summarized in Tables 1 and 2. Specifically, 63.2% of laboratories conduct the test in-house, while 36.8% of laboratories send the samples to other laboratories for testing. The average *EGFR* T790M variant-positive rate (number of *EGFR* T790M variant-positive cases/number of total tested cases) was 10–20% in 42.1% of laboratories.

RECOMMENDATIONS FOR CLINICAL PRACTICE GUIDELINES

To develop clinical practice guidelines, we performed a comprehensive literature search in PubMed, KoreaMed, and Google Scholar for articles in English or Korean related to the pre-analytical phase of *EGFR* variant testing using cfDNA in human subjects. Keyword combinations such as pre-analytical factor, cell-free DNA, cfDNA, ctDNA, *EGFR*, plasma, and liquid biopsy were used. We reviewed the abstracts to shortlist the articles after removing duplicate records. The main content of the shortlisted articles was then reviewed for documentation and are listed in the reference section.

The pre-analytical procedures involved eight steps. Specific recommendations for each step were discussed using evidence from the literature and our experimental data. The recommendations are summarized in Table 3.

Collection of whole blood samples

Cellular DNA is released from lysed white blood cells (WBCs) during the clotting process in serum collection tubes. Consequentially, cellular DNA concentrations are higher in the serum than in the plasma, which may lead to cfDNA becoming diluted with cellular DNA in the serum. Therefore, plasma is a more suitable sample than serum for cfDNA analysis [6, 8, 9].

Tubes with or without cell stabilizer can be used for blood sample collection. For tubes without cell stabilizer, an EDTA tube is recommended and is preferred over other anticoagulants because of the small increase in total DNA when plasma separation is delayed [6, 9–11]. cfDNA analysis using EDTA tubes has been validated previously [6, 11, 12]. It is recommended that sample processing be performed immediately after collection. WBCs are more stable in tubes containing a cell stabilizer, such as the Cell-Free DNA BCT (Streck, La Vista, NE, USA), Cell-Free DNA Collection Tube (Roche Diagnostics), PAXgene Blood ccfDNA Tubes (Qiagen), and Dxtube (Dxome, Seoul, Korea). Thus, if the whole blood cannot be processed within 4–6 hours after collection, tubes with cell stabilizer should be considered [13]. For details on the storage conditions of whole blood before plasma isolation, refer to the section on 'Storage requirements for whole blood'.

In general, the extracted DNA concentration is increased as the plasma input volume is increased [14, 15]. However, over- or under-filling of the blood volume specified by the manufacturer can cause inaccurate test results by altering the blood and additive ratio [16]. For Roche's Cell-Free DNA Collection Tube, Qiagen's PAXgene Blood ccfDNA Tubes, and Dxome Dxtube, the manufacturers suggest 8.5, 10, and 9 mL of whole blood as the optimal sampling volumes, respectively.

Following blood collection, the tubes should be gently inverted

https://doi.org/10.3343/alm.2022.42.2.141

Table 1. Summary of the questionnaire on current practices for plasma *EGFR* variant testing in 19 Korean clinical laboratories

Category	Questions	Answers	N (%) of laboratories
1. Test place, platform, and turn-around time	1.1. Is the test conducted in-house of the institution or outsourced to an external laboratory?	a. In-house	12 (63.2)
		b. External laboratory	7 (36.8)
	1.2. What does the test platform use?	a. Cobas *EGFR* Mutation Test v2	17 (89.5)
		b. PANAMutyper R *EGFR*	0
		c. Both (Cobas and PANAMutyper)	1 (5.3)
		d. No response	1 (5.3)
	1.3. How long does it take to report the test result after a sample is received?	a. Within three days	3 (15.8)
		b. Within seven days	13 (68.4)
		c. Within 10 days	2 (10.5)
		d. No response	1 (5.3)
2. Results of testing	2.1. What is the average monthly *EGFR* T790M variant-positive rate (No. of *EGFR* T790M variant-positive cases/No. of total tested cases)?	a. <10%	3 (15.8)
		b. ≥10 – <20%	8 (42.1)
		c. ≥20 – <30%	2 (10.5)
		d. ≥30 – <40%	1 (5.3)
		e. No response	5 (26.3)
	2.2. What is the optimal *EGFR* T790M variant-positive rate you expect?	a. <10%	1 (5.3)
		b. ≥10 – <20%	8 (42.1)
		c. ≥20 – <30%	3 (15.8)
		d. ≥30 – <40%	1 (5.3)
		e. ≥40 – <50%	2 (10.5)
		f. No response	4 (21.1)
	2.3. Why do you think the actual *EGFR* T790M variant-positive rate is below expectations?*	a. False-negatives due to low tumor burden	8 (42.1)
		b. False-negatives due to limited sensitivity of the test	4 (21.1)
		c. Inadequacy of sample processing (pre-analytical process)	5 (26.3)
		d. Limited number and timing of the test due to criteria of health insurance	4 (21.1)
		e. Small number of tests	4 (21.1)
		f. Other	4 (21.1)
		g. No response	5 (26.3)
3. External quality assessment	3.1. Have you ever participated in external quality assessment programs for plasma *EGFR* variant testing?†	a. Yes	9 (47.4)
		b. No	8 (42.1)
		c. No response	2 (10.5)

*Duplicate answers allowed; †This result is the situation at the time of the survey between December 2018 to January 2019; currently, all laboratories that conduct plasma *EGFR* variant testing participate in external quality assessment programs.
Abbreviations: EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor.

5–8 times immediately to ensure proper mixing of the anticoagulant with the blood. Delayed mixing of blood and anticoagulant can cause the blood to clot and release cellular DNA from the WBCs. Shaking of the tubes should be avoided to prevent hemolysis.

Transport of whole blood samples to laboratory

Following collection, whole blood samples should be transported carefully within the stipulated time frame and with minimal movement and temperature changes as per the sample storage requirement. Agitation of the samples should be avoided to prevent hemolysis and cellular damage, which can lead to cellular DNA release [9, 13].

Storage requirements for whole blood

For EDTA tubes, whole blood should be processed according to the manufacturers' instructions. If there are no specific instructions, whole blood in EDTA tubes should generally be processed within 4–6 hours at room temperature (18°C to 25°C) or 4°C. The whole blood in EDTA tubes should be processed as soon as

Table 2. Summary of the questionnaire on the current practice on the pre-analytical phase of plasma *EGFR* variant testing in 19 Korean clinical laboratories

Object	Questions	Choices	N (%) of laboratories
1. All laboratories (N = 19)	1.1. What blood collection tube do you use?	a. K2 EDTA tube	9 (47.4)
		b. K3 EDTA tube	1 (5.3)
		c. Tubes with cell stabilizer	8 (42.1)
		d. No response	1 (5.3)
	1.2. How much whole blood is collected?	a. 5 mL	3 (15.8)
		b. 5 or 10 mL	1 (5.3)
		c. 6.5 – 8.5 mL	3 (15.8)
		d. 9 mL	2 (10.5)
		e. 10 mL	6 (31.6)
		f. 12 mL	3 (15.8)
		g. No response	1 (5.3)
2. K2 or K3 EDTA tube user (N = 10)	2.1. What is the time interval between blood collection and plasma separation?	a. Within 2 hr	6 (60.0)
		b. Within 4 hr	4 (40.0)
	2.2. How many centrifugation steps do you use to separate the plasma?	a. One	4 (40.0)
		b. Two	6 (60.0)
	2.3. 1st centrifugation method (force and time)	a. ≥1,000 – <2,000 ×*g* for 10 min	2 (20.0)
		b. ≥2,000 – <3,000 ×*g* for 5 min	2 (20.0)
		c. ≥2,000 – <3,000 ×*g* for 10 min	1 (10.0)
		d. ≥3,000 – <4,000 ×*g* for 5 min	2 (20.0)
		e. ≥3,000 – <4,000 ×*g* for 10 min	1 (10.0)
		f. No response	2 (20.0)
	2.4. 2nd centrifugation method (force and time)	a. ≥2,000 – <3,000 ×*g* for 10 min	1 (10.0)
		b. ≥3,000 – <4,000 ×*g* for 10 min	2 (20.0)
		c. ≥10,000 ×*g* for 5 min	1 (10.0)
		d. ≥10,000 ×*g* for 10 min	1 (10.0)
		e. Not done	4 (40.0)
		f. No response	1 (10.0)
	2.5. On average, how long does it take to perform a test after plasma separation?	a. Within a day	1 (10.0)
		b. Within two days	3 (30.0)
		c. Within three days	1 (10.0)
		d. More than three days	5 (50.0)
	2.6. At what temperature do you store separated plasma samples if the test cannot be performed immediately?	a. 2~8°C	1 (10.0)
		b. –20°C	2 (20.0)
		c. –70 ~ –80°C	5 (50.0)
		d. No response	2 (20.0)
3. Users of tubes with cell stabilizer (N = 8)	3.1. Do you perform inversions to mix the sample with anticoagulant and stabilizer solutions after blood collection?	a. Yes	8 (100.0)
		b. No	0
	3.2. Do you know the instructions for storage and plasma processing of your cell stabilizing tubes?	a. Yes	6 (75.0)
		b. No	2 (25.0)

Abbreviation: EGFR, epidermal growth factor receptor.

possible because cellular DNA is released from nucleated cells over time. Previous studies revealed a remarkable increase in the total DNA concentration at 4–6 hours after blood collection [6, 9, 10, 17–21]. Although there is no remarkable difference in sample stability between room temperature and 4°C for a short duration, samples tend to be more stable at 4°C than at room temperature when blood processing is delayed [8, 9, 17, 22].

For tubes containing cell stabilizer, whole blood should be stored according to the manufacturers' instructions. Generally, blood samples in tubes with cell stabilizer should be processed

https://doi.org/10.3343/alm.2022.42.2.141

Table 3. Recommendations for pre-analytical phase of plasma *EGFR* variant testing

Steps	Recommendations
1. Collection of whole blood sample	1.1. Plasma is more suitable than serum for cfDNA analysis.
	1.2. Tubes with or without cell stabilizer can be used. For tubes without cell stabilizer, an EDTA tube is recommended. If processing of whole blood is impossible within 4–6 hr after blood collection, tubes with cell stabilizer should be considered for use.
	1.3. Blood volume should be sufficient to obtain the plasma volume that is recommended in the manufacturers' instructions.
2. Transport of whole blood sample to laboratory	2.1. Hemolysis and agitation of whole blood should be avoided.
	2.2. Whole blood samples should be transported within the proper time duration after blood collection according to the storage requirements of whole blood.
3. Storage requirements for whole blood	3.1. Whole blood in EDTA tubes should be processed within 4–6 hr at room temperature or 4°C.
	3.2. For tubes with cell stabilizer, whole blood should be stored according to manufacturers' instructions.
4. Plasma separation from whole blood	4.1. For plasma isolation, double centrifugation is recommended.
	4.2. Buffy-coat contamination should be avoided.
5. Storage requirements of plasma	5.1. cfDNA should be extracted immediately after separating plasma.
	5.2. For short-term storage, plasma can be stored for 3 hr at 4°C.
	5.3. For long-term storage, plasma should be stored at –20°C or –80°C.
6. Extraction of cfDNA	6.1. Individual laboratories should choose the cfDNA extraction method considering performance, time, and cost.
7. Quality control of cfDNA	7.1. The quantity and quality of extracted cfDNA should be checked before downstream analysis.
8. Storage requirements for cfDNA	8.1. Downstream analysis should be performed immediately.
	8.2. cfDNA should be archived below –20°C.
	8.3. Multiple aliquoting is recommended to avoid multiple freeze–thaw cycles.

Abbreviations: EGFR, epidermal growth factor receptor; cfDNA, cell-free DNA.

within 7–14 days at room temperature. For Cell-Free DNA BCT, the manufacturer suggests a stable storage duration at a temperature of 6–37°C for 14 days, while for the Roche Cell-Free DNA Collection Tube and Dxtube, the manufacturers suggest a stable storage period of seven days at 18–25°C. For PAXgene Blood ccfDNA Tubes, the manufacturer indicates that samples are stable at room temperature (15–25°C) for up to seven days or at higher temperatures (up to 35°C) for up to one day. In previous studies, there was no remarkable difference in sample stability and variant detectability between various commercial tubes with cell stabilizer [23, 24]. In general, it is recommended that plasma is separated as soon as possible after blood is drawn, even when using tubes containing cell stabilizer.

Plasma separation from whole blood

For tubes containing cell stabilizer, whole blood should be processed according to the manufacturers' instructions. The centrifugation conditions proposed by the manufacturers are as follows. For Cell-Free DNA BCT, two separate centrifugation conditions are recommended: 1) first centrifugation at 300 ×*g* for 20 min and second centrifugation at 5,000 ×*g* for 10 minutes; 2) first centrifugation at 1,600 ×*g* for 10 minutes and second centrifugation at 16,000 ×*g* for 10 minutes. For the Roche Cell-Free DNA Collection Tube, a centrifugation condition of 1,900 ×*g* for 15 minutes is recommended. For the PAXgene Blood cfDNA Tubes and Dxtube, double centrifugation at 1,900 ×*g* for 15 minutes and 1,900 ×*g* for 10 minutes is recommended.

As described above, single or double centrifugation is recommended by different manufacturers. Generally, if there are no instructions for tubes containing cell stabilizer or those with EDTA, double centrifugation is recommended to produce cell-free plasma [10]. In single centrifugation, cfDNA can be diluted by cellular DNA from remnant nucleated cells in the plasma.

To compare cfDNA yields and cellular DNA contamination between double and single centrifugations, 20 mL peripheral blood was collected into an EDTA blood collection tube (Vacutainer K2EDTA) (BD Biosciences, Franklin Lakes, NJ, USA). Each 10 mL volume was then split into 2×5-mL aliquots and then incubated at room temperature for two hours. Plasma was isolated under the following two conditions: Centrifugation_Condition_1: first centrifugation at 1,600 ×*g* for 10 minutes and second centrifugation at 3,000 ×*g* for 10 minutes; Centrifuga-

tion_Condition_2: first centrifugation at 1,600 ×*g* for 10 minutes and second high-spin centrifugation at 16,000 ×*g* for 10 minutes. Further, to evaluate the effect of hemolysis on downstream cfDNA concentrations, mechanically hemolyzed whole blood and non-hemolyzed blood were tested under the same conditions (Centrifugation_Condition_1 and Centrifugation_Condition_2). Whole blood samples were mechanically hemolyzed by 2–5 aspirations using a 25-gauge needle before plasma separation [25]. We extracted cfDNA from 2 mL plasma using a MagMAX™ Cell-Free DNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA), and genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen). The distribution and concentration of extracted cfDNA and high-molecular-weight DNA were evaluated using a 2200 TapeStation Instrument with High Sensitivity D1000 ScreenTape and Genomic DNA ScreenTape (Agilent Technologies, Santa Clara, CA, USA).

Short-length DNA (cfDNA) was more abundant when using Centrifugation_Condition_2 (with a second high-spin centrifugation at 16,000 ×*g*) than when using Centrifugation_Condition_1 (with a second low-spin centrifugation at 3,000 ×*g*) for both hemolyzed and non-hemolyzed samples (Supplemental Data Fig. S1). The degree of contamination with high-molecular-weight DNA in cfDNA rarely differed between Centrifugation_Condition_1 and Centrifugation_Condition_2 for non-hemolyzed samples (Supplemental Data Fig. S2A, B). By contrast, high-molecular-weight DNA was more abundant when using Centrifugation_Condition_1 for hemolyzed samples (Supplemental Data Fig. S2C, D). In clinical laboratories where high-spin centrifugation (~16,000 ×*g*) is not available, a second centrifugation performed at 3,000 ×*g* for 10 minutes is recommended [9, 10, 15, 22, 26, 27]. When isolating the supernatant after the first centrifugation, special attention should be paid to avoid contamination by the buffy coat. Therefore, a first centrifugation at 800–1,600 ×*g* for 10 minutes followed by a second high-spin centrifugation at 16,000 ×*g* for 10 minutes is recommended to increase the cfDNA yield and reduce genomic DNA contamination.

Storage requirements of plasma

As cfDNA degradation by nucleases can continue after plasma separation, cfDNA should be extracted immediately from the separated plasma [28]. If the test is not performed immediately, the plasma can be stored for up to three hours at 4°C [9]. For long-term storage, plasma should be frozen at –20°C or –80°C [6]. Because freezing and thawing of plasma cannot be performed more than once, samples should be aliquoted into multiple tubes for subsequent testing [9].

Extraction of cfDNA

The length of cfDNA is shorter than that of genomic DNA [29, 30]. Therefore, cfDNA should be extracted using a method that targets low-molecular-weight DNA. It is recommended to use the cfDNA extraction kit specified in the cell stabilization tubes or *EGFR* variant testing kit. When *EGFR* variant testing is performed using Cobas *EGFR* Mutation Test v2, cfDNA is extracted using the Cobas cfDNA Sample Preparation Kit included in the *EGFR* variant testing kit. For PANAMutyper R *EGFR*, the manufacturer recommends using the QIAamp Circulating Nucleic Acid Kit (Qiagen) for DNA extraction. In addition, several commercial products are available for cfDNA extraction, such as the QIAsymphony Blood ccfDNA Kit (Qiagen), MagMAX Cell Free DNA Isolation Kit (Thermo Fisher Scientific), Maxwell RSC ccfDNA Plasma Kit (Promega, Madison, WI, USA), and Magnetic Serum/Plasma Circulating DNA Kit (Dxome). The QIAamp Circulating Nucleic Acid Kit, QIAsymphony Blood ccfDNA Kit, and Maxwell RSC ccfDNA Plasma Kit can be automated using the QIAcube, QIAsymphony, and Maxwell instruments for DNA extraction, respectively. Individual laboratories can refer to previous studies that have compared the performance of several cfDNA extraction methods or select a method through verification [31–33]. The quantity and quality of extracted cfDNA and time and cost efficiency should be considered when selecting an extraction method.

cfDNA quantification

To quantify cfDNA, spectrophotometry (e.g. NanoDrop), fluorometry (e.g. Qubit), real-time PCR, and digital PCR methods can be used [34]. To quantify low concentrations of cfDNA, spectrophotometry is less accurate than fluorometry [15, 35]. Automatic electrophoresis systems, such as the Bioanalyzer and TapeStation, allow quantification and size measurements of cfDNA.

Storage requirements for cfDNA

Storage conditions for cfDNA should follow the recommendations by the cfDNA isolation kit manufacturer. For the Cobas cfDNA Sample Preparation Kit, extracted cfDNA should be used within the following recommended storage periods: –15°C to –25°C for up to two freeze thaws over 60 days, 2–8°C for up to 21 days, and 15°C or 30°C for up to seven days. For the QIAamp Circulating Nucleic Acid Kit, the recommended storage conditions for eluted cfDNA are 2–8°C for 24 hours and –15°C to –30°C for longer than 24 hours. For the QIAsymphony Blood ccfDNA Kit, the extracted cfDNA is stable at 2–8°C for up to one month and –20°C or –80°C for long-term storage and up to

https://doi.org/10.3343/alm.2022.42.2.141

three freeze-thaw cycles. For the MagMAX Cell-Free DNA Isolation Kit, purified cfDNA can be stored on ice for up to 24 hours and at –20°C for long-term storage.

If there is no recommendation, downstream analysis should be performed immediately. cfDNA should be stored below –20°C. Repeated freeze–thaw cycles can accelerate DNA degradation; thus, multiple aliquots are recommended for subsequent testing. Studies have shown that up to three freeze–thaw cycles of cfDNA do not significantly affect the DNA stability [9, 20].

This survey was conducted when plasma *EGFR* variant testing was initially covered by the National Medical Insurance System of Korea, which may differ from the current practice. Among the 12 institutions that conducted the test in-house, nine had participated in the external quality assessment (EQA) programs at least once. Currently (in 2020), 28 institutions are participating in the EQA program conducted by the Korea Association of External Quality Assessment Service. The number of institutions currently conducting plasma *EGFR* variant testing would be higher than that at the time of conducting the survey.

The T790M variant-positive rate in the plasma of patients who develop progressive disease from prior EGFR-TKI therapy has been reported to be 17.5%–48.1% depending on the testing methods [36–38]. Although we did not divide the T790M variant-positive rate according to the previous history of EGFR-TKI therapy, the results in a high proportion (73.7%) of responders revealed that the actual T790M variant-positive rate was lower than expected. Various factors may affect the T790M variant-positive rate, including patient factors, test sensitivity, and limited criteria of health insurance. Currently, in Korea, plasma *EGFR* variant testing can only be performed once at disease progression if changes in the treatment regimen are necessary. However, repeat plasma testing after a negative initial test may be beneficial when tissue is not available for molecular testing or when a negative result is obtained. Previous studies also reported that additional follow-up plasma testing can detect *EGFR* T790M variants in a significant number of patients following an initial T790M-negative blood test result [39].

Further, pre-analytical factors, such as transport time, plasma volume, centrifugation protocol, and sample storage, would account for a decreased T790M variant-positive rate. Seven of the 19 (36.8%) institutions answered that they send samples for plasma *EGFR* variant testing to an external laboratory. To ensure accuracy of test results, laboratories should manage the sample transport time and conditions. Four of the 10 institutions using the EDTA tubes answered that they do not perform double centrifugation for plasma separation, which, in the case of single centrifugation, can affect the test results by failing to remove cell debris [14]. After training and applying the recommendations proposed in this article, the survey should be repeated to observe the resulting changes.

CONCLUSION

In conclusion, these clinical practice guidelines will serve to standardize the pre-analytical procedures of plasma *EGFR* variant testing in clinical laboratories. These guidelines do not address cfDNA analysis using body fluids, such as pleural fluid, bronchoalveolar lavage fluid, and cerebrospinal fluid. However, body fluids other than plasma have been reported as promising sources for *EGFR* variant testing [40, 41]. Thus, guidelines for pre-analytical procedures for body fluids will be needed in the future.

ACKNOWLEDGEMENTS

None.

AUTHOR CONTRIBUTIONS

Shin S and Woo HI reviewed the literature and wrote the original draft. Kim JW provided advice. Kim Y and Lee KA designed and supervised the study and reviewed and edited the manuscript.

CONFLICTS OF INTEREST

No potential conflicts of interest relevant to this article are reported.

RESEARCH FUNDING

This study was supported by the Research Fund of Quality Control Committee, Korean Society for Laboratory Medicine (KSLM Research Project 2020-01-006) and Korean Society for Genetic Diagnostics.

ORCID

Saeam Shin	https://orcid.org/0000-0003-1501-3923
Hye In Woo	https://orcid.org/0000-0002-9332-7058
Jong-Won Kim	https://orcid.org/0000-0002-0708-9242
Yoonjung Kim	https://orcid.org/0000-0002-4370-4265
Kyung-A. Lee	https://orcid.org/0000-0001-5320-6705

REFERENCES

1. Langer CJ. Epidermal growth factor receptor inhibition in mutation-positive non-small-cell lung cancer: is afatinib better or simply newer? J Clin Oncol 2013;31:3303-6.
2. Chae H, Sung PS, Choi H, Kwon A, Kang D, Kim Y, et al. Targeted Next-Generation Sequencing of Plasma Cell-Free DNA in Korean Patients with Hepatocellular Carcinoma. Ann Lab Med 2021;41:198-206.
3. Ministry of Health and Welfare of South Korea. Notice of safety and efficacy of new medical device. https://www.mohw.go.kr/react/jb/sjb-0406vw.jsp (Updated on Oct 2017).
4. Li X and Zhou C. Comparison of cross-platform technologies for EGFR T790M testing in patients with non-small cell lung cancer. Oncotarget 2017;8:100801-18.
5. Passiglia F, Rizzo S, Di Maio M, Galvano A, Badalamenti G, Listì A, et al. The diagnostic accuracy of circulating tumor DNA for the detection of EGFR-T790M mutation in NSCLC: a systematic review and meta-analysis. Sci Rep 2018;8:13379.
6. Meddeb R, Pisareva E, Thierry AR. Guidelines for the preanalytical conditions for analyzing circulating cell-free DNA. Clin Chem 2019;65:623-33.
7. Underhill HR, Kitzman JO, Hellwig S, Welker NC, Daza R, Baker DN, et al. Fragment length of circulating tumor DNA. PLoS Genet 2016;12:e1006162.
8. Jung M, Klotzek S, Lewandowski M, Fleischhacker M, Jung K. Changes in concentration of DNA in serum and plasma during storage of blood samples. Clin Chem 2003;49:1028-9.
9. El Messaoudi S, Rolet F, Mouliere F, Thierry AR. Circulating cell free DNA: Preanalytical considerations. Clin Chim Acta 2013;424:222-30.
10. Normanno N, Denis MG, Thress KS, Ratcliffe M, Reck M. Guide to detecting epidermal growth factor receptor (EGFR) mutations in ctDNA of patients with advanced non-small-cell lung cancer. Oncotarget 2017;8:12501-16.
11. Lam NY, Rainer TH, Chiu RW Lo YM. EDTA is a better anticoagulant than heparin or citrate for delayed blood processing for plasma DNA analysis. Clin Chem 2004;50:256-7.
12. van Dessel LF, Beije N, Helmijr JC, Vitale SR, Kraan J, Look MP, et al. Application of circulating tumor DNA in prospective clinical oncology trials - standardization of preanalytical conditions. Mol Oncol 2017;11:295-304.
13. Medina Diaz I, Nocon A, Mehnert DH, Fredebohm J, Diehl F, Holtrup F. Performance of Streck cfDNA blood collection tubes for liquid biopsy testing. PLoS One 2016;11:e0166354.
14. Sherwood JL, Corcoran C, Brown H, Sharpe AD, Musilova M, Kohlmann A. Optimised pre-analytical methods improve KRAS mutation detection in circulating tumour DNA (ctDNA) from patients with non-small cell lung cancer (NSCLC). PLoS One 2016;11:e0150197.
15. van Ginkel JH, van den Broek DA, van Kuik J, Linders D, de Weger R, Willems SM, et al. Preanalytical blood sample workup for cell-free DNA analysis using Droplet Digital PCR for future molecular cancer diagnostics. Cancer Med 2017;6:2297-307.
16. Deans ZC, Williams H, Dequeker EMC, Keppens C, Normanno N, Schuuring E, et al. Review of the implementation of plasma ctDNA testing on behalf of IQN Path ASBL: a perspective from an EQA providers' survey. Virchows Arch 2017;471:809-13.
17. Xue X, Teare MD, Holen I, Zhu YM, Woll PJ. Optimizing the yield and utility of circulating cell-free DNA from plasma and serum. Clin Chim Acta 2009;404:100-4.
18. Angert RM, LeShane ES, Lo YM, Chan LY, Delli-Bovi LC, Bianchi DW. Fetal cell-free plasma DNA concentrations in maternal blood are stable 24 hours after collection: analysis of first- and third-trimester samples. Clin Chem 2003;49:195-8.
19. Lui YY, Chik KW, Chiu RW, Ho CY, Lam CW, Lo YM. Predominant hematopoietic origin of cell-free DNA in plasma and serum after sex-mismatched bone marrow transplantation. Clin Chem 2002;48:421-7.
20. Chan KC, Yeung SW, Lui WB, Rainer TH, Lo YM. Effects of preanalytical factors on the molecular size of cell-free DNA in blood. Clin Chem 2005;51:781-4.
21. Jeong T-D, Kim MH, Park S, Chung H-S, Lee JW, Chang JH, et al. Effects of pre-analytical variables on cell-free DNA extraction for liquid biopsy. Lab Med Online 2019;9:45-56.
22. Risberg B, Tsui DWY, Biggs H, Ruiz-Valdepenas Martin de Almagro A, Dawson SJ, Hodgkin C, et al. Effects of collection and processing procedures on plasma circulating cell-free DNA from cancer patients. J Mol Diagn 2018;20:883-92.
23. Alidousty C, Brandes D, Heydt C, Wagener S, Wittersheim M, Schäfer SC, et al. Comparison of blood collection tubes from three different manufacturers for the collection of cell-free DNA for liquid biopsy mutation testing. J Mol Diagn 2017;19:801-4.
24. Warton K, Yuwono NL, Cowley MJ, McCabe MJ, So A, Ford CE. Evaluation of Streck BCT and PAXgene stabilised blood collection tubes for cell-free circulating DNA studies in plasma. Mol Diagn Ther 2017;21:563-70.
25. Lee J-H, Kim Y, Yoo G, Kim J, Yoon KJ, Uh Y. Algorithm for differential diagnosis of in vitro and in vivo hemolysis in anticoagulated blood specimens. J Lab Med Qual Assur 2017;39:132-40.
26. Chiu RW, Poon LL, Lau TK, Leung TN, Wong EM, Lo YM. Effects of blood-processing protocols on fetal and total DNA quantification in maternal plasma. Clin Chem 2001;47:1607-13.
27. Swinkels DW, Wiegerinck E, Steegers EA, de Kok JB. Effects of blood-processing protocols on cell-free DNA quantification in plasma. Clin Chem 2003;49:525-6.
28. Sanchez C, Snyder MW, Tanos R, Shendure J, Thierry AR. New insights into structural features and optimal detection of circulating tumor DNA determined by single-strand DNA analysis. NPJ Genom Med 2018;3:31.
29. Heitzer E, Ulz P, Geigl JB. Circulating tumor DNA as a liquid biopsy for cancer. Clin Chem 2015;61:112-23.
30. Kim Y, Shin S, Kim B, Lee KA. Selecting short length nucleic acids localized in exosomes improves plasma EGFR mutation detection in NSCLC patients. Cancer Cell Int 2019;19:251.
31. Pérez-Barrios C, Nieto-Alcolado I, Torrente M, Jiménez-Sánchez C, Calvo V, Gutierrez-Sanz L, et al. Comparison of methods for circulating cell-free DNA isolation using blood from cancer patients: impact on biomarker testing. Transl Lung Cancer Res 2016;5:665-72.
32. Sorber L, Zwaenepoel K, Deschoolmeester V, Roeyen G, Lardon F, Rolfo C, et al. A comparison of cell-free DNA isolation kits. J Mol Diagn 2017;19:162-8.
33. Markus H, Contente-Cuomo T, Farooq M, Liang WS, Borad MJ, Sivakumar S, et al. Evaluation of pre-analytical factors affecting plasma DNA analysis. Sci Rep 2018;8:7375.
34. Trigg RM, Martinson LJ, Parpart-Li S, Shaw JA. Factors that influence quality and yield of circulating-free DNA: A systematic review of the methodology literature. Heliyon 2018;4:e00699.
35. Ponti G, Maccaferri M, Manfredini M, Kaleci S, Mandrioli M, Pellacani G, et al. The value of fluorimetry (Qubit) and spectrophotometry (NanoDrop) in the quantification of cell-free DNA (cfDNA) in malignant melanoma and prostate cancer patients. Clin Chim Acta 2018;479:14-9.
36. Zheng D, Ye X, Zhang MZ, Sun Y, Wang JY, Ni J, et al. Plasma EGFR

T790M ctDNA status is associated with clinical outcome in advanced NSCLC patients with acquired EGFR-TKI resistance. Sci Rep 2016;6:20913.
37. Wei B, Zhao C, Li J, Zhao J, Ren P, Yang K, et al. Combined plasma and tissue genotyping of EGFR T790M benefits NSCLC patients: a real-world clinical example. Mol Oncol 2019;13:1226-34.
38. Chen YL, Lin CC, Yang SC, Chen WL, Chen JR, Hou YH, et al. Five technologies for detecting the EGFR T790M mutation in the circulating cell-free DNA of patients with non-small cell lung cancer: a comparison. Front Oncol 2019;9:631.
39. Keedy VL, Temin S, Somerfield MR, Beasley MB, Johnson DH, McShane LM, et al. American Society of Clinical Oncology provisional clinical opinion: epidermal growth factor receptor (EGFR) mutation testing for patients with advanced non-small-cell lung cancer considering first-line EGFR tyrosine kinase inhibitor therapy. J Clin Oncol 2011;29:2121-7.
40. Hummelink K, Muller M, Linders TC, van der Noort V, Nederlof PM, Baas P, et al. Cell-free DNA in the supernatant of pleural effusion can be used to detect driver and resistance mutations, and can guide tyrosine kinase inhibitor treatment decisions. ERJ Open Res 2019;5:00016-2019.
41. Li YS, Jiang BY, Yang JJ, Zhang XC, Zhang Z, Ye JY, et al. Unique genetic profiles from cerebrospinal fluid cell-free DNA in leptomeningeal metastases of EGFR-mutant non-small-cell lung cancer: a new medium of liquid biopsy. Ann Oncol 2018;29:945-52.