EQA for cfDNA molecular analysis of *EGFR* in Lung cancer 2018

On behalf of the IQN Path cfDNA EQA group
(a sub-group of the Liquid Biopsy Working Group)

Summary Report

Contents

14th November, 2019

Dear Colleague,

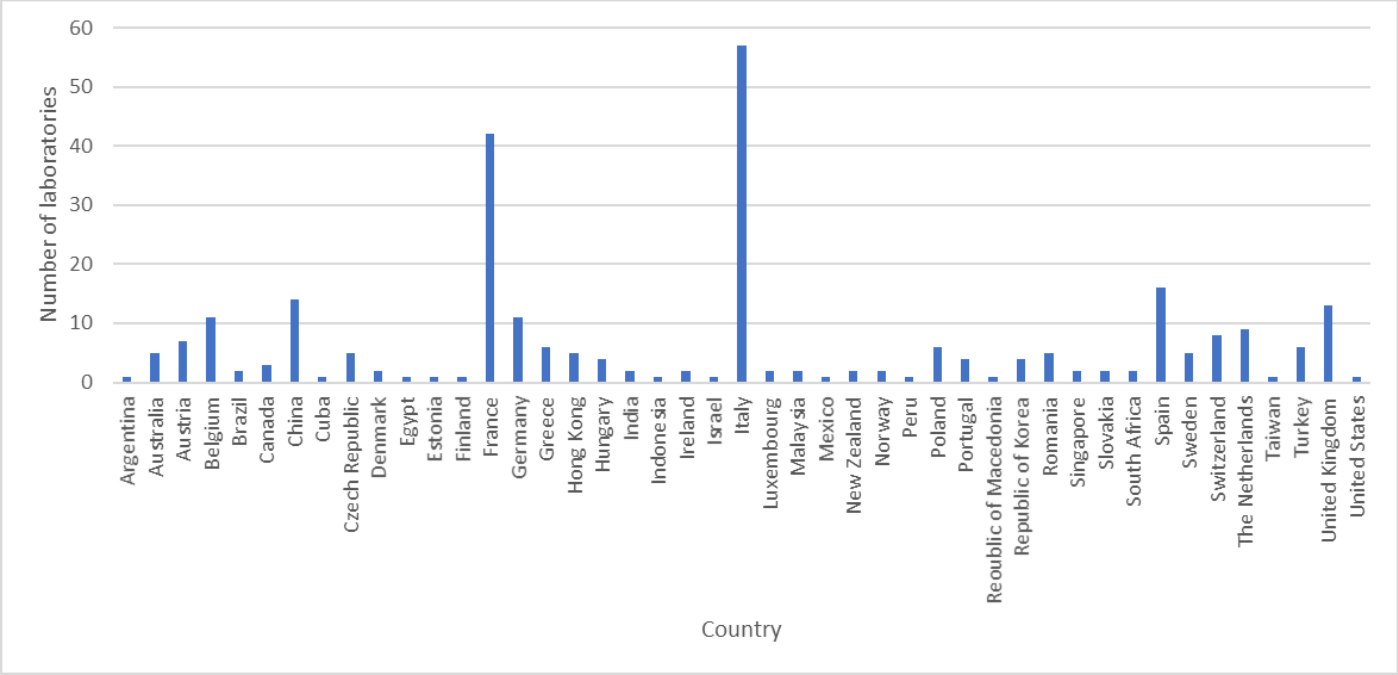
Thank you for participating in the external quality assessment (EQA) 2018 to assess the standard of testing for the presence of *EGFR* mutations in circulating cell-free DNA (cfDNA) in plasma. This EQA has been provided as an IQN Path collaboration between five External Quality Assessment (EQA) providers (AIOM, EMQN, ESP EQA Gen&Tiss and GenQA).

The harmonised marking has been completed for all EQA providers and your individual laboratory scores were released to you by your own EQA provider. Each EQA provider has issued their laboratory scores and an EQA Summary Report. This report is an overarching summary which collates the results from all EQA providers and discusses the issues raised by the assessors during the marking process.

1. Participation

Three hundred and four laboratories from 45 different countries registered with the five EQA providers to participate in this EQA (see Figure 1). Two hundred and sixty-four (87%) laboratories submitted results for the EQA. Two of the EQA providers are national Schemes (France and Italy) therefore participation was from a single country.

Figure 1 *– Summary of geographical location of laboratories*



2. Samples provided and testing required

The EQA was designed to enable laboratories to test plasma cfDNA for the presence of *EGFR* variants. Five samples were distributed for testing and all participating laboratories regardless of which EQA provider they participated through were supplied with the same samples. Testing was to be performed according to normal laboratory procedures and the findings to be reported using the laboratory's routine format. The samples were artificially manufactured to contain 250ng of cfDNA and each sample was supplied as 3mL of synthetic plasma.

During the distribution of these samples a small number of laboratories reported leaking of the plasma from the tubes during transportation. Most of the laboratories reporting these leaking samples were supplied with replacements to ensure they were not disadvantaged by sub-optimal EQA material. Auditing of the EQA results from these

laboratories confirmed that the leaking samples did not cause any issues as the laboratories concerned reported the correct genotyping results. However, five laboratories reported the leakage in their reports submitted to their EQA provider and had not requested repeat samples.

Auditing of the EQA returns identified that all laboratories using the Diatech Pharmacogenetics Helix® Circulating Nucleic Acid kit were unable to obtain cfDNA suitable for testing using the samples provided for this EQA. The EQA providers have investigated and identified that the issue was related to the manufactured samples. In the future, IQNPath will try to ensure that the samples provided for this EQA will be suitable for all extraction methods.

3 Clinical cases

A clinical case scenario was provided for each of the five EQA samples (Table 1). Participants were required to submit fully interpretative reports tailored to answer the clinical question detailed in the scenarios.

Table 1 *– Summary of clinical cases and expected results*

Sample	Case	Patient details	Reason for referral	Genotype
IQN Path Sample 2018 – A	1	Elena NOVELLO (dob 02/05/1956) Female	Never smoker patient, diagnosed with metastatic lung adenocarcinoma at age 62. *EGFR* testing performed on the patient's tumour biopsy specimen failed. Testing for *EGFR* gene mutations on the patient's plasma sample has been requested.	c.2236_2250del p.(Glu746_Ala750del) (1.3% allelic fraction)
IQN Path Sample 2018 – B	2	Sara CIMINO (dob 10/11/1937) Female	Patient with metastatic lung adenocarcinoma diagnosed at age 80. After resection, tumour tissue was analysed and no *EGFR* variant was detected. *EGFR* gene testing has been requested on the patient's plasma sample.	No mutations detected within regions tested
IQN Path Sample 2018 – C	3	Ferdinand GARCIA (dob 18/08/1947) Male	Patient with metastatic lung adenocarcinoma, diagnosed at age 68. Patient received first line EGFR-TKI treatment and is now in clear clinical progression. No tissue sample or cytology specimen of progressing disease is available due to their poor clinical condition. Testing of the patient's plasma sample for *EGFR* gene variants has been requested.	c.2369C >T p.(Thr790Met) (5.1% allelic fraction) and c.2573T>G p.(Leu858Arg) (4.7% allelic fraction)
IQN Path Sample 2018 – D	4	David CLARKE (dob 03/10/1962) Male	Patient diagnosed with metastatic lung adenocarcinoma at age 55. The patient was found to have an *EGFR* mutation and received first line treatment with an EGFR-TKI. At progression of the disease on TKI, the patient had a tissue biopsy but no tumour cells were present. *EGFR* gene testing has been requested on the patient's plasma sample	c.2236_2250del p.(Glu746_Ala75del) (6.2% allelic fraction)
IQN Path Sample 2018 – E	5	Adele HOLMES (dob 29/09/1952) Female	Patient diagnosed with *EGFR*-mutant metastatic lung adenocarcinoma at age 65. The patient has a radiological progression of their primary tumour wheras the metastatic lesions are stable. Testing for *EGFR* gene variants on patient's plasma sample has been requested.	c.2369C>T p.(Thr790Met) (0.81% allelic fraction) and c.2573T>G p.(Leu858Arg) (0.49% allelic fraction)

Key dob: date of birth

4. Validated results

All samples were validated by five independent laboratories prior to distribution and the results are summarised in Table 2.

Table 2 *- Validated results*

	Sample					Method
	A	B	C	D	E	
Expected results	**c.2236_2250del p.(Glu746_Ala750del) (1.3%)**	**WT**	**c.2369C>T p.(Thr790Met) (5.1%) and c.2573T>G p.(Leu858Arg) (4.7%)**	**c.2236_2250del p.(Glu746_Ala750del) (6.2%)**	**c.2369C>T p.(Thr790Met) (0.81%) and c.2573T>G p.(Leu858Arg) (0.49%)**	
Laboratory 1	Ex19del (unspecified)	WT	c.2369C>T p.(Thr790Met) c.2573T>G; p.(Leu858Arg)	Ex19del (unspecified)	c.2573T>G p.(Leu858Arg)	Therascreen® ™ EGFR Plasma RGQ PCR Kit (Qiagen)
	c.2236_2250del p.(Glu746_Ala750del) (1.11%)	WT	c.2369C>T p.(Thr790Met) (4.46%) c.2573T>G p.(Leu858Arg) (5.10%)	c.2236_2250del p.(Glu746_Ala750del) (6.26%)	c.2369C>T p.(Thr790Met) (0.66%) c.2573T>G p.(Leu858Arg) (0.33%)	Oncomine™ Lung cfDNA Assay (Life Technologies) on S5XL System (Life Technologies)
	c.2236_2250del p.(Glu746_Ala750del) (1.17%)	WT	c.2369C>T p.(Thr790Met) (4.56%) c.2573T>G p.(Leu858Arg) (3.72%)	c.2236_2250del p.(Glu746_Ala750del) (2.62%)	c.2573T>G p.(Leu858Arg) (1.35%)	GeneRead™ QIAact Lung UMI Panel (Qiagen) on GeneReader NGS System (Qiagen)
Laboratory 2	Ex19del (unspecified)	WT	c.2369C>T p.(Thr790Met) c.2573T>G p.(Leu858Arg)	Ex19del (unspecified)	c.2573T>G; p.(Leu858Arg)	Stilla ddPCR technologies
Laboratory 3	Ex19del (unspecified) (SQI 12.22)	WT	c.2369C>T p.(Thr790Met) (SQI 12.95) c.2573T>G p.(Leu858Arg) (SQI 7.99)	Ex19del (unspecified) (SQI 14.43)	c.2369C>T p.(Thr790Met) (SQI 9.23)	cobas® EGFR Mutation Test v2 (Roche)
Laboratory 4	N/A	N/A	c.2369C>T p.(Thr790Met) (SQI 15.52) c.2573T>G p.(Leu858Arg) (SQI 8.99)	N/A	c.2369C>T p.(Thr790Met) (SQI 10.21)	cobas® EGFR Mutation Test v2 (Roche)
	Ex19del (unspecified) (2.7%)	WT	c.2369C>T p.(Thr790Met) (4.5%) c.2573T>G p.(Leu858Arg) (5.2%)	Ex19del (unspecified) (5.6%)	c.2369C>T p.(Thr790Met) (0.65%) c.2573T>G p.(Leu858Arg) (0.59%à	ddPCR (BioRad-assays)
Laboratory5	N/A	N/A	c.2369C>T p.(Thr790Met) (SQI 14.28) c.2573T>G p.(Leu858Arg) (SQI 10.2)	N/A	c.2369C>T p.(Thr790Met) (SQI 9.55)	cobas® EGFR Mutation Test v2 (Roche)

Key N/A: Not analysed, WT: wild type (no mutation detected)

Nomenclature according to *EGFR* gene reference sequences NM_005228.4 or LRG_304t1.

For some of the techniques used for the validation testing, the mutations were below the known limits of detection (LOD) of the assay and therefore would not be expected to be reported.

The validation results for case 5 were inconsistent with regards to the detection of the presence of the *EGFR* c.2573T>G p.(Leu858Arg) variant in the sample. See section 5 for further discussion. In particular only two of the validation techniques used (Oncomine™Lung cfDNA assay and Bio-Rad ddPCR assay) were able to consistently detect both mutations expected to be present in this case.

5. Assessment process

Participants were expected to submit interpretative reports for assessment of genotyping accuracy. In addition, a review of the interpretation of the results and the clerical accuracy of the submitted reports was performed. Assessors' feedback comments were provided when required.

In line with other EQA schemes provided by AIOM, EMQN, ESP EQA, Gen&Tiss and GenQA, the genotyping was assigned a maximum score of 2.0 marks and the marking criteria outlined in Table 3 were applied. Interpretation was also marked by EMQN, ESP EQA, Gen&Tiss and GenQA and assigned 2.0 marks with the criteria outlined in Table 3 applied. The assessment of performance was only applied to the genotyping accuracy.

Table 3 *– Marking criteria*

Marking Category	Criterion	Deduction
Genotyping	Correct result reported (method enables mutation characterisation AND correct HGVS nomenclature used)	0
	Correct result reported (method does not enable mutation characterisation)	0
	Correct result reported within the limitations of the test performed	0
	False positive result in any gene reported (critical genotyping error)	2
	False negative result reported (mutation is present below limit of detection or reporting cut-off of assay)	0
	False negative result reported (not known if mutation is present below limit of detection or reporting cut-off of assay: critical genotyping error)	2
	Incorrect mutation reported (critical genotyping error)	2
	Only 1 mutation reported (for cases with 2 mutations and limit of detection or reporting cut off below variant frequency; critical genotyping error)	2
	Mutation described incorrectly e.g. incorrect deletion reported at nucleotide or amino acid level (non-critical genotyping error)	0.5
	No (or incorrect) HGVS nomenclature used	0.5
	Minor HGVS error	0
	Technical failure (see comments)	1
	Mutation reported at protein level not nucleic acid level	0.5
	Method performed is able to characterise the mutation but the characterised result has not been reported	0.5
	SNP reported but not identified as SNP	0.5
Interpretation	All essential interpretative elements provided	0
	Critical interpretation error	2
	Misleading interpretive comment	1
	It is recommended to state that the analysis of a plasma sample is not 100% sensitive and therefore the presence of a mutation may have been missed (refers to cases 2 and 4 only)	0

	Over / inappropriate interpretation of a negative (or normal) results using cfDNA. For example, advising that the absence of the mutation indicates that the patient would be unlikely to benefit from an EGFR TKI (refers to cases 2 and 4 only)	0.5
	Incorrect sample type reported (e.g. FFPE instead of Plasma)	0.2
	No statement about the methodology performed	0.5
	Failure to provide any, or insufficient, details of the scope of the test and/or limitations of the test performed, in relation to the suitability of the material provided.	0.2
	Insufficient information provided on the NGS testing methodology - platform, and/or manufacturer, and/or strategy (i.e. WES, targeted) not listed on report	0.2
	Incorrect and/or inconsistent use of patient name affecting meaning of report	1
	LRG or RefSeq missing / incorrect / inconsistent	0.5 (marks deducted once)
Clerical Accuracy	All essential patient identifiers present and no significant clerical errors.	0
	Date of birth incorrect (any error)	1
	Patient name has minor spelling error	0.5
	Incorrect or missing patient gender	0
	Failure to provide sample reference number	0.5
	Report confusing or difficult to read - essential information "hidden" within the body of the text. It is recommended that critical pieces of information are highlighted in some way so that they are not overlooked.	0
	Report confusing or difficult to read - too many pages and/or unnecessary use of images.	0
	Pagination should be used on the report e.g. Page 1 of 1, Page 1 of 2 etc.	0

The three validation laboratories using the cobas™ assay did not detect the presence of the c.2573T>G p.(Leu858Arg) in case 5. Furthermore, of the 72 laboratories using cobas™ in the EQA itself, only a small number reported the presence of this mutation. It was therefore decided by the EQA Providers that laboratories would not be deducted marks for failing to detect this mutation in this caseTherefore, the following criteria were applied (see Table 4).

Table 4 *– Criteria applied to Case 5*

Reported result	Genotyping deduction	Comment to laboratories
Both mutations detected	0	Correct result
c.2369C>T p.(Thr790Met) only detected	0	This sample also contained a c.2573T>G p.(Leu858Arg) mutation at approximately 0.5%. Please see scheme report for further details.
c.2573T>G p.(Leu858Arg) only detected and LOD for c.2369C>T p.(Thr790Met) is below 0.8%	1	This sample also contained a c.2369C>T p.(Thr790Met) mutation at approximately 0.8%. Please see scheme report for further details.
c.2573T>G p.(Leu858Arg) only detected and LOD for c.2369C>T p.(Thr790Met) is above 0.8%	0	This sample also contained a c.2369C>T p.(Thr790Met) mutation at approximately 0.8%. Please see scheme report for further details.
No mutations detected and LOD for both is below 0.5% for c.2573T>G p.(Leu858Arg) and 0.8% for c.2369C>T p.(Thr790Met)	1	This sample contained both a c.2573T>G p.(Leu858Arg) mutation at approximately 0.5% and a c.2369C>T p.(Thr790Met) at approximately 0.8% and the stated limits of detection of your assay indicate that both mutations should have been detected.

No mutations detected and LOD for both is above 0.5% for c.2573T>G p.(Leu858Arg) and 0.8% for c.2369C>T p.(Thr790Met)	0	This sample contained both a c.2573T>G p.(Leu858Arg) mutation at approximately 0.5% and a c.2369C>T p.(Thr790Met) at approximately 0.8%. Please see scheme report for further details.

6. Appeals process

Laboratories were given the opportunity to appeal deductions made by their individual EQA providers in this EQA. Across all EQA providers, a total of 14 (5%) laboratories submitted appeals for this EQA.

In summary:

- Four laboratory appeals were upheld and the marks returned;
- One laboratory appeal was partially upheld and a proportion of the marks returned;
- One laboratory appeal was upheld but this did not result in a change to the score;
- Eight laboratory appeals were rejected.

7. Scheme report on behalf of the assessors

The following issues were highlighted by the EQA assessors during the marking process.

7.1 Genotyping accuracy

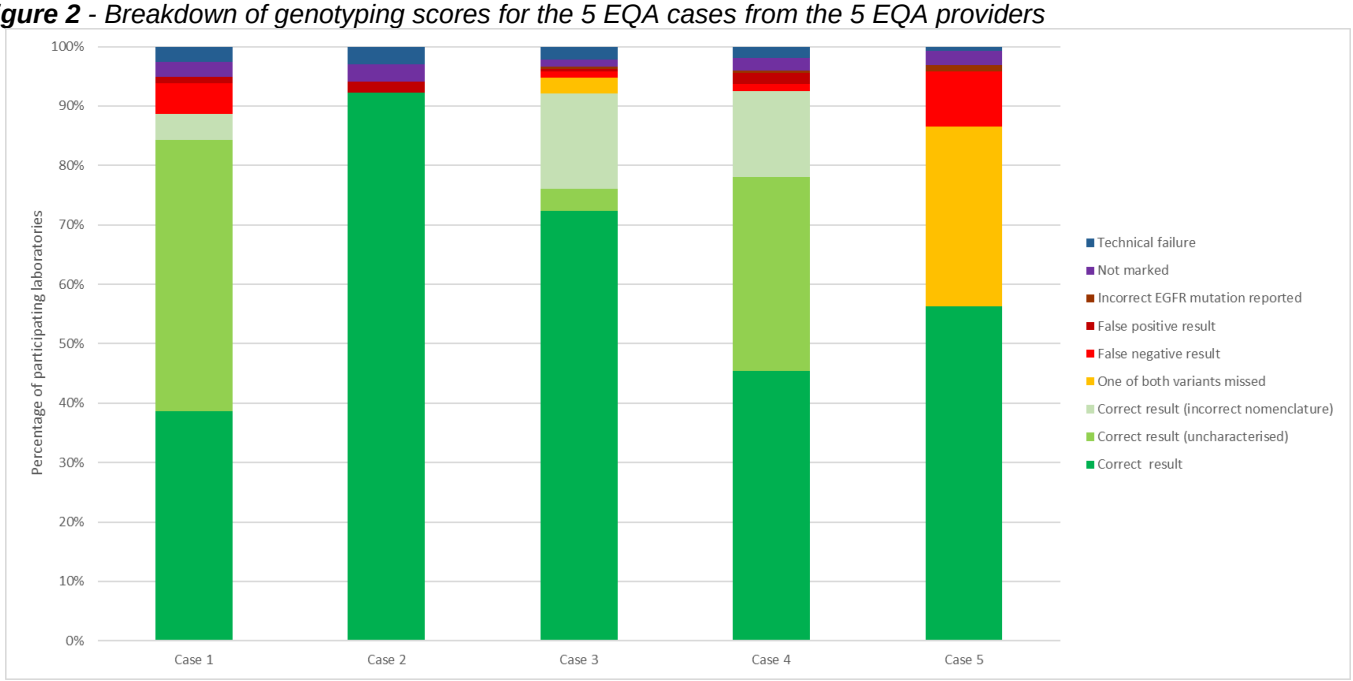
Table 5 displays the average scoring for each EQA sample across all EQA providers and Figure 2 details the breakdown of scoring.

Table 5 *- Mean scores of all participating laboratories (maximum score = 2)*

Category	Case 1	Case 2	Case 3	Case 4	Case 5
Mean Genotyping Score	1.83	1.93	1.82	1.86	1.83
Mean Interpretation Score*	1.58	1.45	1.54	1.40	1.49
Mean Clerical Accuracy Score*	1.89	1.9	1.9	1.89	1.89

* EMQN, ESP, Gen&Tiss and GenQA data only

Figure 2 *- Breakdown of genotyping scores for the 5 EQA cases from the 5 EQA providers*



Overall the results for cases 1, 2, 3 and 4 were of a very high standard with more than 85% of laboratories reporting the correct results. By comparison only 147 (56%) of laboratories reported the correct result for case 5.

Case 1 and 4 both contained the same deletion in exon 19 of *EGFR* and there were a number of laboratories which used incorrect nomenclature to describe the mutations; 12 (5%) of laboratories in case 1 and 39 (15%) of laboratories in case 4. This resulted in deductions from the laboratories' genotyping scores. A substantial proportion of laboratories were unable to characterise the mutations in these cases; 125 (46%) of laboratories for case 1 and 8 (33%) of laboratories for case 4. This was related to the technology used for testing which did not enable the mutation present to be fully characterised, and therefore these laboratories were not penalised.

There were also a number of laboratories which reported either one or both of the mutations in case 3 using incorrect nomenclature and this resulted in deductions from the laboratories' genotyping scores.

For cases 1, 2, 3 and 4 there were only a small number of laboratories reporting incorrect results

- For case 1 there were 14 laboratories which reported false negative results and three who reported false positives;
- For case 2 there were five laboratories which reported false positive results. This was a case where no mutations were present in *EGFR;*
- For case 3 there were three laboratories reporting false negative results,one laboratory which reported a false positive and a further one laboratory which reported an incorrect *EGFR* mutation. An additional seven laboratores missed one of the variants but reported the other correctly;
- For case 4 there were five laboratories which reported a false positive results, three which reported a false negative reults and one laboratory which reported an incorrect muation.

Case 5 was a more challenging sample with two mutations present in *EGFR:* c.2369C>T p.(Thr790Met) at 0.81% allelic frequency and c.2573T>G p.(Leu858Arg) at 0.49% allelic frequency. Due to issues with the validation of this sample and the fact that only 56% of laboratories reported the correct result, the marking criteria was adjusted for this case as detailed in Table 5. Twenty-four (9%) laboratories missed both mutations and 79 (33%) laboratories reported the presence of only one mutation. Of those laboratories which only reported one mutation, 62 (23%) of these only reported the presence of the c.2369C>T mutation in *EGFR*. In addition, there were three laboratories which reported an incorrect *EGFR* mutation in this case.

7.2 Reporting of results

Several laboratories used the terms 'positive/negative' to describe the mutation status in reports, which can be misinterpreted. It is recommended to use 'mutation detected/mutation not detected' instead.

7.2.1 Mutation nomenclature using HGVS (Human Genome Variation Society) v19.01

The majority of laboratories described the mutations using HGVS nomenclature however there were some laboratories which only reported the results at the amino acid level. As the assays used are DNA based tests, then the mutations found should be described in terms of the nucleotide change(s) and ideally with the predicted amino acid changes also provided. Although some of the commercial kits used to perform *EGFR* tests report the results only at the amino acid level it is possible to determine the nucleotide changes from the literature provided with the kit.

7.2.2 Use of reference sequences

Gene reference sequences should be included in reports even if commercial kits are being used (and do not reference genes within the kit inserts). Even when a laboratory is using a commercially available assay to perform a test, it should be possible to determine the reference sequence for the gene(s) tested and the individual EQA providers gave information regarding reference sequences in the EQA distribution letter/Scheme Instructions.

The following paper demonstrates the importance of using a standardised nomenclature and appropriate reference sequence for reporting mutation results, Tack V, Deans ZC, Wolstenholme N, Patton S, Dequeker EMC. 2016. What's in a name? A coordinated approach towards the correct use of a uniform nomenclature to improve patient reports and databases. Hum Mutat 37:570–575.

7.2.3 Sample type

A small number of laboratories reported the incorrect type of sample tested e.g. FFPE instead of plasma. We remind participants that it is important to accurately describe the sample tested. When using reporting templates it is important that they are updated when a new test is implemented, e.g. using solid tumour reporting templates which have not been fully adapted for ctDNA testing.

7.3 Report Format

During the assessment process, it was noted that it was not always easy to find the essential information within the body of the text. Many reports embedded essential information e.g. the clinical interpretation either in a non-prominent position on the report or as a general statement in in the footer or as a rider. It is recommended that critical pieces of information are highlighted in some way so that they are not overlooked by the clinician reading the report.

7.4 Reporting 'no mutation detected' results

Several laboratories over-interpreted a "no mutations detected" result, advising that the absence of a mutation indicated that the patient would be unlikely to respond to EGFR TKIs. This interpretation is inappropriate and more caution is advised as cfDNA analyses are known to have reduced sensitivity for detecting mutations. In the event of a "no mutation detected" result, a repeat sample should be indicated or a clear statement regarding the reduced sensitivity of plasma testing and the risk of no cfDNA being present in the sample tested.

7.5 Testing methodologies

The majority of laboratories provided details of the testing they performed and compared to the previous IQN Path pilot cfDNA EQA in 2-17 there was an improvement in the details provided of Next Generation Sequencing (NGS) methodology. Data was also collected regarding the DNA extraction methodology used by laboratories, using the survey provided and by examining the submitted reports however, not all laboratories provided this information. Figures 3, 4 and 5 summarise the different methods performed for DNA extraction and *EGFR* mutation testing.

Figure 3 *– Methodologies performed for DNA extraction*

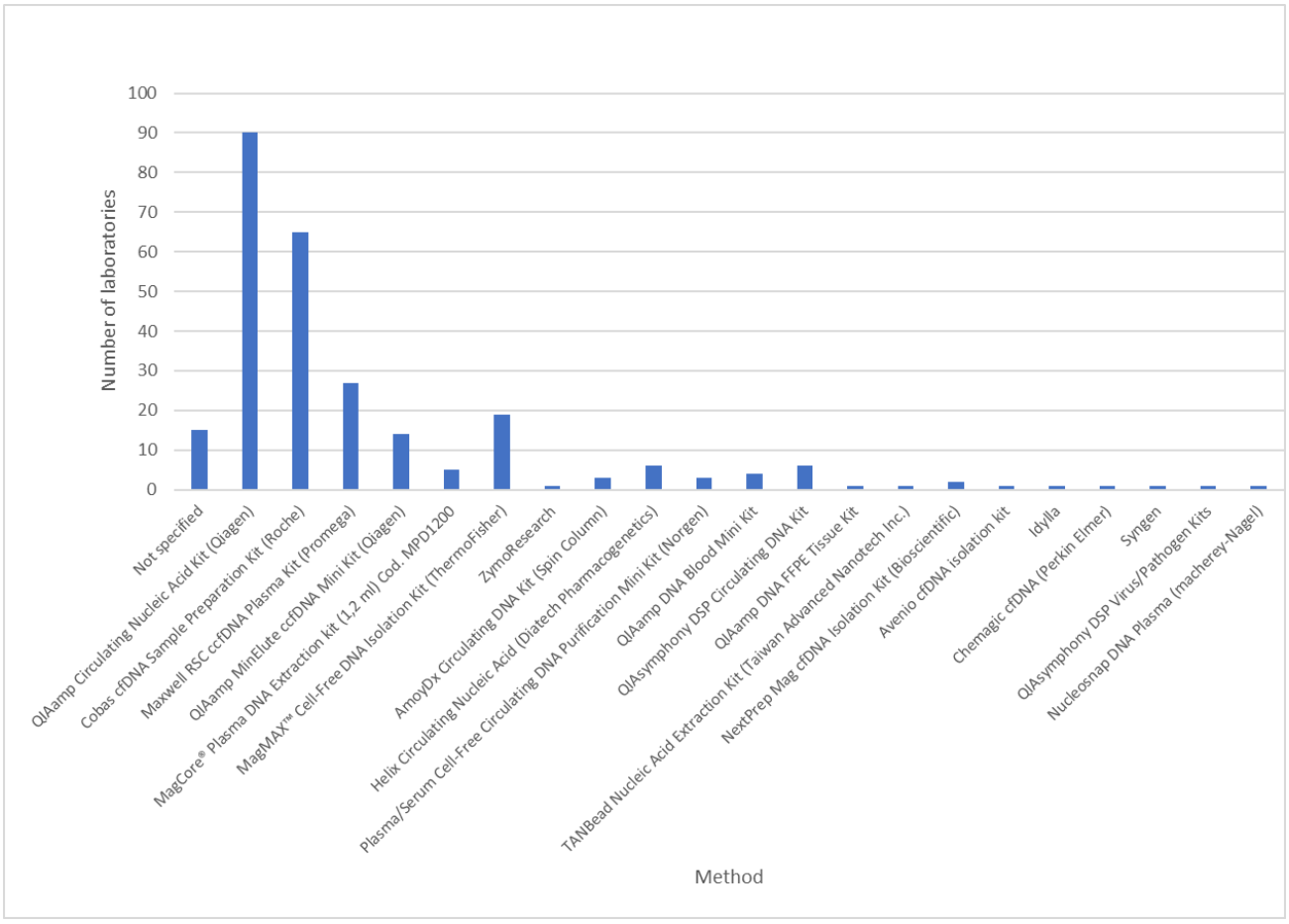


Figure 4 *– Methodologies performed for EGFR mutation testing*

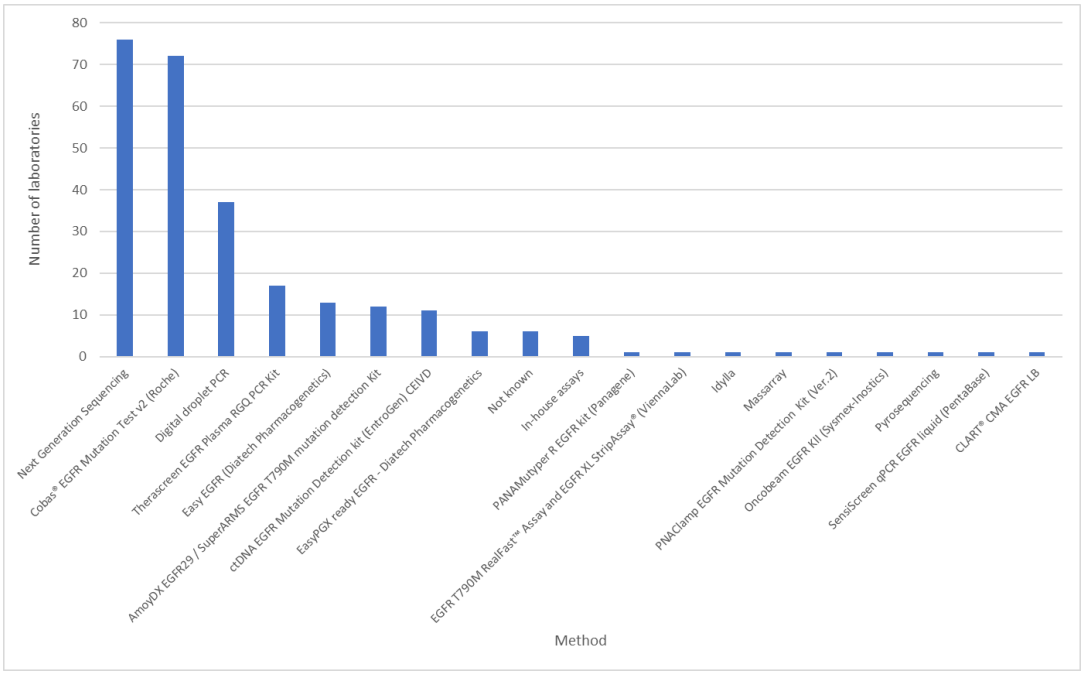
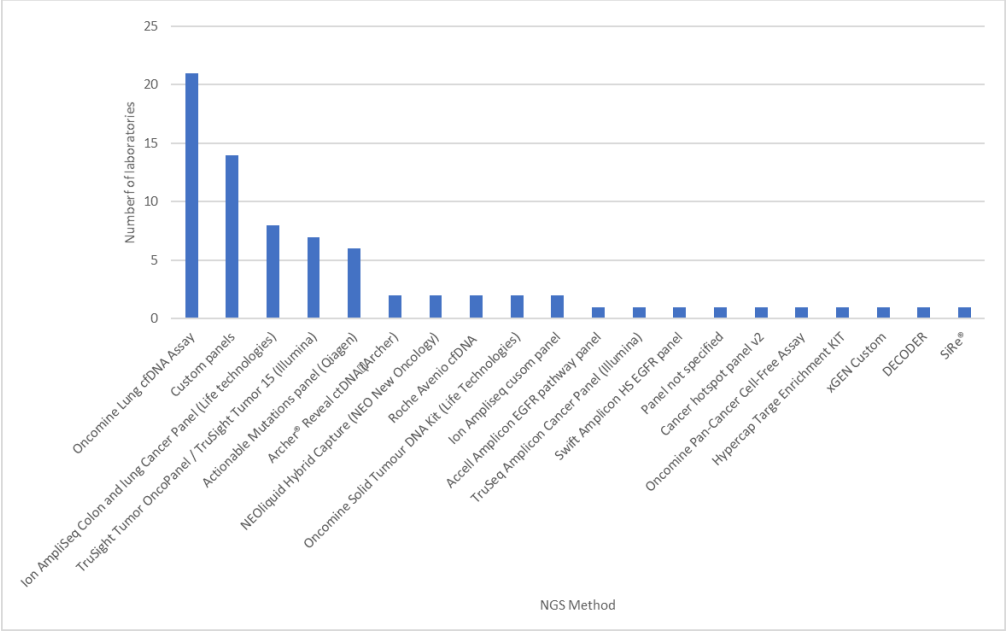


Figure 5 *– Breakdown of NGS Methodologies performed for EGFR mutation testing*



7.6 Testing limitations

As observed in the IQN Path pilot EQA delivered in 2017, some reports provided insufficient information on the limitations of the test performed and the following marking was applied:

- If no mutation was detected but the limit of detection (LOD) was less than the variant allelic frequency, then no deduction was applied.
- If no mutation was detected and no LOD was stated on the report then this was classed as a genotyping error and a deduction made.

Interpreting the reported allelic frequency is often difficult and there is a need to standardise the units used to measure/report variant allelic frequencies. Laboratories used either copies/ml or a percentage. These measurements

are not equivalent and for some testing methods it would be appropriate and good practice to use both. The percentage value for limits of detection gives an indication of how sensitive an assay is at detecting a mutation in a background of wild type DNA. The copies/ml will give an indication of the minimum number of copies of DNA an assay can detect. Ideally both of these values should be determined during the validation process prior to the introduction of the test into routine clinical practice. It is recognised that for NGS it is not possible to determine the sensitivity in copies/ml due to the technology used.

7.7 Scope of testing

There were a number of laboratories which performed limited *EGFR* mutation testing i.e. only testing for the c.2369C>T p.(Thr790Met) variant. In the case of first line treatment for anti-EGFR tyrosine kinase inhibitors (as in cases 1 and 2) it is inappropriate to only test for the c.2369C>T p.(Thr790Met) variant as this will not provide sufficient information for a clinician to determine the possibility of response to treatment. In the case of progressive disease, it is good practice to test for the original *EGFR* variant identified as this can provide useful data on the sensitivity of the assay performed i.e. if a resistance mutation is not detected and the original variant is not detected consideration must be given to the likelihood of insufficient test sensitivity..

One laboratory did not provide results for cases 1 and 2 as they would not perform plasma testing in the context of first line treatment.

8. Future distribution

A full EQA will be provided in 2019-20. Laboratories will be contacted by their respective EQA providers when registration is open. There will be no restriction on participant numbers.

Thank you for participating in this EQA and we hope you have found it a useful exercise. If you require any further information, then please contact your EQA scheme provider directly.

Kind regards,

GenQA, ESP QA Foundation, AIOM/SIAPEC, Gen&Tiss, and EMQN
(see next page for more information)

Authorisation/Approval

This document has been authorised / approved on behalf of each EQA provider by:

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- Gustave Roussy, Villejuif, France
- Laboratoire de Biochimie, CHU, Nantes, France
- Manchester Centre for Genomic Medicine, Manchester United Kingdom