



EGFR PCR/Sequencing Guidance Notes

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N.B. This document is a summary of best practice and methods for EGFR mutation detection by PCR/Sequencing based on shared experiences and published data. Where unreferenced, these are the recommendations based on the experience of AstraZeneca scientists. These recommendations are for laboratories that request advice from AstraZeneca on sequencing methodology. This document provides information that will allow increased assay success rates on patient material, eliminate the chances of false positive and false negative results, and if required provide a mechanism of standardisation of testing practices across laboratories. These recommendations are not meant to be prescriptive. It may not be feasible for some laboratories to adopt all of the measures described. In addition laboratories may have their own best practices that work well and do not require alteration.

1 Abbreviations

ARMS	Amplification Refractory Mutation System
AZ	AstraZeneca
BSA	Bovine Serum Albumin
CDNA	Coding DNA
COSMIC	Catalogue of Somatic Mutations in Cancer
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide Triphosphate
EGFR	Epidermal Growth Factor Receptor
FFPET	Formalin Fixed Paraffin Embedded Tissue
NSCLC	Non Small Cell Lung Cancer
O.D.	Optical Density
PCR	Polymerase Chain Reaction
Q-PCR	Quantitative Polymerase Chain Reaction
SOP	Standard Operating Procedure
TKI	Tyrosine Kinase Inhibitor
UV	Ultra Violet

2 Introduction

2.1 EGFR Mutations

Mutation analysis of the EGFR kinase domain can be carried out using a variety of methods including PCR/Sequencing, ARMS, pyrosequencing. PCR/Sequencing is a commonly used method, however, there is a great deal of variation in the detail of how this methodology is carried out. The aim of this document is to provide best practice guidelines on PCR/Sequencing for EGFR mutation analysis.

A spectrum of mutations exists within the EGFR kinase domain in tumours of patients with Non Small Cell Lung Cancer (NSCLC). The most frequently observed mutations are the Exon 19 deletions and the Exon 21 L858R mutation, which taken together account for ~85%-90% of all EGFR mutations (Lynch *et al.*, 2004; Paez *et al.*, 2004; Sharma *et al.*, 2007; Hirsch and Bunn, 2009). These mutations are associated with sensitivity to EGFR tyrosine kinase inhibition (TKI). Others including T790M, where currently there is little or no data supporting sensitivity to EGFR TKIs (Mok *et al.*, 2008; Kim *et al.*, 2008; Hirsch *et al.*, 2006; AZ In-House Data - Unpublished). There are numerous less frequent mutations where the data is still emerging. The full spectrum of mutations reported in the literature can also be found in Sanger's COSMIC Database ([Link](#)).

2.2 Importance of Robust Methodology in EGFR Analysis

The number of steps involved in EGFR mutation analysis is dependent on the platform used. Shown in Figure 1 is an example workflow for EGFR mutation analysis by dye-terminator sequencing. No matter what method used, the analysis is highly dependent on both the quality of the sample and the DNA extracted from it.

The material available for mutation testing in most cases is Formalin Fixed Paraffin Embedded Tissue (FFPET). This fixation process followed by long term storage of blocks and/or slides results in fragmentation of the DNA, and as a result, difficulty in analysing DNA extracted from these samples. The damage caused to the DNA by the formalin fixation process can result in the generation of artefacts during the PCR process, which manifest themselves as non-reproducible sequence alternations. It is essential that these non-reproducible sequence alterations be differentiated from 'true' mutations by confirmatory analysis (Eberhard *et al.*, 2008; Kimura *et al.*, 2006).

In addition, tumour samples are heterogeneous. It is possible that cells containing mutant DNA may make up only part of the total cells present. Normal cells will also be present in the sample despite macrodissection. It is for this reason that sensitive methods of mutation detection may be employed to determine the mutation status of the sample.

Following robust procedures, such as those described in section 2 of this document will maximise the likelihood of obtaining a true mutation result through

- Pathology review to ensure sample integrity
- DNA extraction to ensure sufficient material for analysis
- Mutation analysis procedures to ensure assay success
- Robust quality control procedures to ensure reporting of accurate mutation status

(Eberhard et al., 2008; AZ In-House Experience - Unpublished)

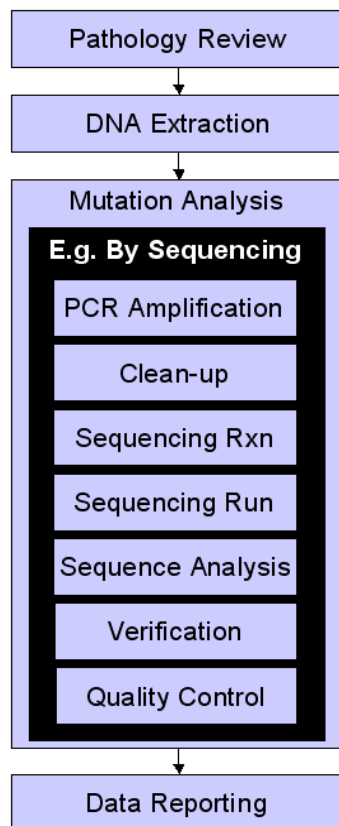


Figure 1: Workflow for EGFR Mutation Analysis by Sequencing (AZ- unpublished)

3 Methodology: Recommendations and Best Practice

3.1 Pathology Review of Samples Prior to Mutation Analysis

(AZ In-House Experience, Unpublished and Eberhard *et al.*, 2008)

Key messages

- ⇒ Pathology review is required to ensure that tumour cells are present
- ⇒ If possible, macrodissection should be carried out
- Pathological review of tumour samples provided for EGFR mutation analysis is vital to ensure the quality of the sample is sufficient for the analysis to occur. Without assessing the quality of the sample, the inclusion of poor quality samples will increase the likelihood of “false negative” results. In addition, pathological review also provides the opportunity to gather important descriptive data on the nature of the samples provided.
- If possible, the area containing these tumour cells should be marked-up on the slide by the pathologist.
- Macrodissection (e.g. scraping using a scalpel) prior to DNA extraction will result in enrichment of tumour DNA, and therefore less contaminating normal DNA will be encountered due to the potential heterogeneity of the tumour (Eberhard *et al.*, 2008). Macrodissection will reduce the chances of obtaining false negative result.
- A pathologist may want to record the following information
 - Sample Form: block or slides
 - Sample Type: biopsy or resection
 - Tissue: Lung, lymph node, pleura, metastatic site etc.
 - Adequate tissue: >50-100 cells?
 - Diagnosis: e.g. adenocarcinoma
 - Tumour Grade: well, moderately or poorly differentiated
 - Other: Including inflammatory, necrotic, mitotic, apoptotic cells etc.

3.2 DNA Extraction

(AZ In-House Experience, Unpublished and Eberhard *et al.*, 2008)

Key Messages:

- ⇒ The quality and quantity of DNA extracted from a sample is key to downstream analysis. A poor DNA extraction method can lead to increased assay failures, increase the chance of false positive and false negative results
- ⇒ DNA quantification is optional, but maybe useful for prioritising exons for screening and for troubleshooting assay failures
- Extraction methods should be robust and reliable chosen so as to yield as much DNA as possible, while minimising carry-over of inhibitors that may interfere with downstream applications. There are numerous methods available for DNA extraction from tumour tissue. Popular methods include kit-based methods such as QIAamp® DNA Mini Kit (Qiagen) [Link](#), Chelex based methods, amongst others.
- Determination of DNA concentration via O.D. readings is not always accurate, as this will measure all DNA in the sample, not only the amplifiable DNA. Quantification of DNA samples by Q-PCR is a superior method, as only the amplifiable DNA will be measured hence providing an accurate measure of the amount of DNA available for analysis. Samples with DNA yields <2 copies/μl of amplifiable DNA will often fail analysis. However, in these cases the recommendation is to amplify Exons 19 and Exons 21 initially so as to increase the chances of detecting the common EGFR mutations.

3.3 PCR Amplification of the EGFR Kinase Domain

(AZ In-House Experience, Unpublished; Paez *et al.* 2004, and Lynch *et al.* 2004, Eberhard *et al.*, 2008)

Key messages:

- ⇒ Methods and reagents used for PCR amplification should be chosen to produce successful amplification in as many samples as possible
- ⇒ The use of primers to amplify small regions of DNA~150-200bp rather than large amplicons 200-400bp will result in increased assay success
- ⇒ Paez *et al.* (2004), and Lynch *et al.* (2004) provide primer sequences that may be utilised. Alternatively, laboratories may design and utilise alternative primers, but ensure that they perform well in terms of assay success and quality of product i.e. no non-specific amplification.
- ⇒ The presence of mutations should be verified in independent PCR amplification reactions to avoid reporting of false positive results.
- ⇒ Novel mutations should be distinguished from germline polymorphisms
- ⇒ Several laboratories use nested PCR for amplification of key exons within EGFR. This may be considered if assay success with one-step PCR is thought to be an issue
- ⇒ For both single-step and nested PCR, the risk of contamination should be considered. Steps to avoid contamination should be taken. This includes the use of separate pre- and post-PCR rooms, and the use of positive and negative control reactions throughout the process

3.3.1 PCR Amplification (Regular and Nested)

- Once a patient's DNA sample has been obtained, PCR amplification of the sample will be carried out. It is recommended that the sample be amplified in two independent PCR reactions. If this is not possible due to cost issues and/or a limited supply of DNA, then amplify in a single reaction (however, note the importance of verification of mutations, section below).
- If a sample or batch of samples fails to amplify, dilute the sample 1 in 2, 1 in 5 and/or 1 in 10. It is possible that any inhibitors carried over from the DNA extraction process will then be diluted out. Alternatively, add BSA.
- Prepare DNA dilutions of patient samples if required, in distilled, nuclease free water (except if the DNA concentration is low, then the sample should be amplified undiluted).
- Prepare control DNAs prior to amplification. A no template control (water) and positive control DNA (mutation positive cell-line DNA and mutation negative DNA).
- Perform PCR amplification using an available instrument with optimised cycling conditions.
- Once PCR amplification is complete, products should be run out on an Agarose Gel e.g. 2%. Visualise by UV transillumination and photograph. Successful and failed PCR reactions should be recorded.
- If performing nested PCR, repeat amplification process using appropriate primers, nested PCR reaction mixes and cycling conditions, visualising PCR products on an agarose gel as before.

3.3.2 PCR Product Clean-up

- Following PCR amplification, excess primers, dNTPs and other PCR reagents must be removed from the reaction so as not to interfere with the sequencing reaction. There are numerous methods available which utilise enzymatic (e.g. ExoSAP-IT [Link](#)) or column based methods.
- Following clean-up, an aliquot of the product will go forward for sequencing. The remainder should be stored at -20°C should further analysis be required.

3.4 DNA Sequencing

(AZ In-House Experience, Unpublished; Paez *et al.* 2004, and Lynch *et al.* 2004, Eberhard *et al.*, 2008)

Key Messages:

- ⇒ Sequencing reactions (dye-terminator reactions) should be carried out in a similar manner to PCR amplification. Positive and negative controls should be included and steps should be taken to avoid any risks of contamination
- ⇒ Sequencing instruments vary widely. Sequencing runs on capillary sequencers should be carried out according to robust SOPs. Sequencing instrumentation should be well maintained
- Processes and procedures vary according to sequencing chemistry and instrumentation employed. One of the most commonly used chemistries is that from Applied Biosystems [Link](#), on instruments from the same company. Please refer to the most appropriate method for the instrument(s) employed in your laboratory.
- The most common platform for sequencing comes from Applied Biosystems. The attached sequencing guide provides detail on the procedures that should be followed.

3.5 Analysing Sequence Traces

(AZ In-House Experience, Unpublished; Paez *et al.* 2004, and Lynch *et al.* 2004, Eberhard *et al.*, 2008, Applied Biosystems Website)

Key Messages:

- ⇒ Reading of sequencing traces can be carried out in two ways, by-eye and through the use of sequencing software. The results of sequence analysis by one operator (by eye or with a software package) should always be verified by an independent operator. Operators should be well trained and be mindful of what they are looking for. Any inconclusive results should be discussed prior to reporting.
- ⇒ There are numerous software packages available for sequence analysis including Mutation Surveyor (Soft Genetics [Link](#)) and SeqScape (Applied Biosystems [Link](#)). Whichever package is used, settings should be altered so as to pick up potentially low-level somatic mutations. All results should be analysed by two independent operators.
- ⇒ Appropriate reference sequences should be used so that any variants found are named appropriately (nucleotide and amino acid).
- ⇒ If using sequencing software, settings should be set lower than that usually used for the detection of gene polymorphisms in germline DNA

3.5.1 Basic Procedure for Sequence Analysis using Mutation Surveyor (SoftGenetics [Link](#))

- Mutation Surveyor is currently commonly used for sequence analysis and will be described in detail below. Although the process will differ with other packages, the principles will be the same. Further detail can be found in the Mutation Surveyor User Manual and Help Section within the software.
- Import all sequencing traces into the software package as described in the user manual using the relevant GenBank file as a reference file.
- Perform the sequencing analysis and alignments, ensuring that the 2D small peak option is selected to maximise sensitivity for under-represented sequences.
- Save three versions of the Mutation Surveyor Project. One to remain unedited for reference, the other two being the working files to be analysed by independent operators.
- The sequence may be trimmed so that analysis of only the sequence of interest is carried out, e.g. 10 base pairs of flanking intronic sequence.
- Sequence traces should be analysed by checking all variants against the reference sequence, as highlighted by the sequencing software (several laboratories will check all sequences by eye, in addition. This is optional).
 - False positive calls should be deleted.
 - True positive calls should be marked as “confirmed”.
- The nomenclature of the true calls should be checked thoroughly
 - If a mutation is identified that is the same as a previously identified mutation e.g. in Figure 1, then this should add to confidence that this is a real mutation.
 - If a novel mutation, not previously described in Figure 1, or Sanger’s COSMIC database [Link](#), then this should be questioned. Firstly, the variant should be checked as to whether it is a germline (host) polymorphism. If yes, report as a germline, or non-somatic variant. If the variant is not a known polymorphism, the presence of this mutation should be verified.
- Notes of Caution during sequence analysis
 - Sequence analysis software can miss deletions if the percentage mutant sequence in wild type background is below a certain threshold (<5%).
 - Software may call a string of overlapping peaks after a deletion/insertion in the forward direction, or before a deletion/insertion in the reverse direction. If a string of mutations is seen the analyst should check the data for deletions and insertions by eye.
 - Check the read length of each trace, if it is shorter than the exon delete the sample and classify as a sequencing failure.
- Each operator should compare outputs. Any discrepancies should be discussed and resolved prior to a final copy of the output report being produced. In this report the designated naming convention must be used for all polymorphisms/mutations i.e. base pairs numbered relative to the reference cDNA sequence and amino acids relative to the complete reference protein sequence.

NB: There are several packages available for sequence analysis. The ability of those to detect mutations should be tested. This can be done by reading traces by eye initially prior to relying solely on the software.

3.6 Verification of Mutation Status

(AZ In-House Experience, Eberhard *et al.*, 2008)

Key Messages:

- ⇒ If a variant is observed its presence must be verified in an independent PCR reaction.
 - ⇒ If the possibility of a rare host polymorphism is considered, DNA from patient blood sample could be analysed to eliminate the possibility of reporting a non-tumour variant
- Samples will only be deemed mutation positive if the variant has been observed in at least two independent PCR/Sequencing reactions, and the variant is not a germline polymorphism, i.e. not a tumour or somatic mutation. Table 1 below, is a decision grid for the requirements of mutation verification, prior to reporting.
 - It is not necessary to have both forward and reverse data for a sample if the mutation is confirmed in an independent PCR/Sequencing reaction.
 - If a variant is identified in only one PCR/Sequencing reaction, and there is insufficient DNA to perform an additional independent amplification of the sample, then classify as “Fail” for mutation analysis, and obtain additional sample.
 - The dangers of not adhering to this include
 - Reporting of artefacts known to be an issue in the analysis of FFPE derived DNA
 - Reporting of PCR errors
 - Reporting of rare germline polymorphisms, not somatic mutations, which are not relevant in this area
 - An alternative approach is to verify a mutation using an alternative method.

Table 1: Recommended Decision Grid for Verification of Mutation Status (AZ-unpublished)

Observation	Known EGFR Mutation?	Further Verification Required?	Action Required? Comment
1 of 3 reactions (Forward and Reverse)	Yes	Yes	Yes. Confirm in at least 2 independent PCR reactions
	No	Yes	Yes. Confirm in at least 2 independent PCR reactions, and check if this is a polymorphism
2 of 3 reactions (Forward and Reverse)	Yes	No	No, confident result is true
	No	Yes	Yes. Check if this is a polymorphism
3 of 3 reactions (Forward and Reverse)	Yes	No	No, confident result is true
	No	Yes	Yes. Check if this is a polymorphism

3.7 Quality Control and Quality Procedures

(AZ In-House Experience)

- All processes should be carried out using established laboratory Standard Operating Procedures (SOPs).
- Quality Control of processes and results should be carried out, prior to reporting of mutation data.
- Trained operators should carry out analysis according to these SOPs.
- Laboratory records should be complete and should meet any local and regional requirements.
- Facilities should be of the required standard, ensuring separation between pre-and post-PCR area.
- Secure areas for sample and document management should be available.
- Equipment and instrumentation and refrigeration/freezers should be well maintained (with maintenance logs).
- Reagents should be clearly marked with expiry dates.
- A quality audit of laboratories in regions could be considered which may incorporate lab inspections, inspections of particular processes, or quality monitored through concordance testing of sample sets in multiple labs.

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