TT04. Anglo-American Ring Trial Unveils Pre-PCR Error and False-Negative Results in BRAF and EGFR Clinical Cancer Diagnostics

J.R. Kapp¹, R.A. Hamoudi¹, T. Diss¹, I. Schrijver², L.J. Jennings³, M. Li⁴, G.J. Tsongalis⁵, D. de Castro⁶, J. Bridge⁷, R. Butler⁸, A. Wallace⁹, S. Hing¹⁰, J. Spicer¹¹, E. Verghese¹², G. Latham¹³

¹UCL Cancer Institute, London, Londonshire, England; ²Stanford University Medical School, Stanford, CA; ³Children's Hospital of Chicago, Chicago, IL; ⁴Baylor College of Medicine, Houston, TX; ⁵Dartmouth Hitchcock Medical Center, Lebanon, NH; ⁶Institute of Cancer Research & Royal Marsden Hospital, London, England; ⁷University of Nebraska Medical Center, Omaha, NE; ⁸All Wales Genetics Laboratory, Cardiff, Wales; ⁹St Mary's Hospital, Manchester, Macher; ¹⁰Great Ormond Street Hospital for Children, London, England; ¹¹Guy's Hospital, London, England; ¹²Leeds Institute of Molecular Medicine, Leeds, United Kingdom; ¹³Asuragen, Austin, TX.

Introduction: Treating cancer patients with targeted kinase inhibitors relies on mutation testing employing sequencing methods that have improved exponentially in sensitivity, yet considering the association between pre-PCR and analytical accuracy, it is perhaps surprising that pre-PCR efficiency has not been systematically assessed in the clinical setting. In the present study, we employed novel FFPE reference materials containing pre-defined quantities of DNA, in turn harboring defined abundances of BRAF and EGFR mutations, to assess pre-PCR methods.

Methods: We recruited 13 leading pathology centers from the US and UK and distributed a total of 108 blinded FFPE specimens. The FFPE specimens were classed into 8 types. Types 1 to 4 were cell-line derived sections. Types 1 and 2 carried EGFR p.G719S at 33%, BRAF p.V600E at 25% and EGFR p.L858R at 20%, BRAF p.V600E at 66% respectively. Types 3 and 4 carried corresponding wild-type alleles. Type 5 was a blank section devoid of amplifiable DNA. Type 6 comprised serial sections from a primary tonsil biopsy. Types 7 and 8 were duplicates of 1 and 2. Each laboratory processed all 8 specimens according to their protocols. Participants returned specimens 1 to 6 for follow-up analysis, whilst specimens 7 and 8 continued for sequencing.

Results: Twelve of 13 centers reported nanodrop spectrophotometry for DNA quantitation. Self-reported measurements of DNA yields from 78 cell-line FFPE specimens were compared against expected and theoretical yields, resulting in an average recovery of 130.1% (95% CI, 104.7% to 155.4%) compared with theoretical yields, and an average recovery of 260.2% (95% CI, 209.5% to 310.9%) compared with expected yields. Surprisingly, an average yield of 235.8 nanograms (95% CI, 90.7 ng to 380.9 ng) was reported for the blank FFPE specimens. A total of 37 of 42 potential calls accurately reported the presence of mutations, with the 5 missed calls representing a false-negative rate of 11.9%.

Conclusions: Follow up measurements with Qubit revealed a systematically lower value compared to NanoDrop. Across all labs, the median NanoDrop/Qubit ratio was 5. Nanodrop users may wish to apply this ratio to normalise their values. Qubit measurements highlighted rather extraordinary disparities from lab-to-lab compared to NanoDrop. In conclusion, over quantitation of DNA yield compared to theoretical output was observed in relation to Nanodrop. A false-negative analytical rate approaching 12% was also associated with variation in quantitation, providing evidence for a causal relationship between pre-PCR and analytical accuracy. Further investigation and awareness is required to safeguard patients from errors stemming from pre-PCR analysis.

H34. Detection of EZH2 Mutations in Follicular and Diffuse Large B-Cell Lymphomas Using PCR Followed by High Resolution Melting Curve Analysis

A. Mahmoud¹, J.M. Gale², M.A. Vasef³

¹University of New Mexico Health Science, Albuquerque, NM; ²University of New Mexico/TriCore Ref Lab, Albuquerque, NM.

Introduction: EZH2 methyltransferase is highly expressed in germinal center-derived B-cells and is required for formation of germinal centers. Somatic mutations of EZH2 gene may enhance germinal center formation and in cooperation with BCL2 may contribute to lymphomagenesis via silencing of target genes. A recurrent gain of function mutation involving codon 641 of EZH2 gene has been identified in
folicular lymphomas (FL) and diffuse large B-cell lymphomas (DLBCL) of germinal center (GC) origin. More recently, it has been shown that inhibition of EZH2 suppresses the proliferation of EZH2-mutant cell lines derived from DLBCL. There is inconsistency in the prevalence of reported EZH2 mutations that range from 7% to 22% in folicular lymphomas and 14% to 22% in DLBCL of GC-origin. In this study, we analyzed the incidence of EZH2 mutations on DNA extracted from several cases of germinal center cell lymphoma as well as activated B-cell lymphoma using PCR followed by a rapid high resolution melting curve analysis. Methods: Formalin-fixed paraffin embedded tissue blocks from 38 cases with diagnosis of non-Hodgkin lymphoma were retrieved from archived file of Department of Pathology at the University of New Mexico. The cases included 17 FL, 18 DLBCL, 1 composite DLBCL and T-cell lymphoma, and 2 lymphomatoid granulomatosis. Among the DLBCL cases, 11 were GC-derived and 7 were activated B-cell phenotype (ABC) according to the Hans algorithm. DNA was isolated from diagnostic paraffin blocks and was PCR amplified using primers flanking the codon 641 of exon 15 of the EZH2 gene. The PCR products were then subjected to high resolution melting curve analysis (Idaho Technologies). Results: High resolution melting curve analysis demonstrated an abnormal melting curve with a shouldering in 4 out of 38 cases including 3 out of 17 (18%) FL cases and 1 out of 11 (9%) of GC-derived DLBCL. The Y641H mutation was confirmed by Sanger sequencing. Conclusions: Our results demonstrate that high resolution melting technology is a useful and rapid method to screen and detect EZH2 mutations in non-Hodgkin B-cell lymphomas. In addition, the results of our study confirm the prior reported higher incidence of EZH2 mutations in cases of FL. However, the prevalence of EZH2 mutation in GC-derived DLBCL in our study is lower than the one reported previously (9% versus 14% to 22%). This might be due to smaller number of cases in our study. Similar to prior studies, we did not identify EZH2 mutations in ABC type DLBCL cases.

ST30. Clinical Next-Generation Sequencing: Automation of Result Interpretation and Reporting Workflow
S. Roy, M.B. Durso, A. Wald, Y.K. Ng, Y.E. Nikiforov, M.N. Nikiforova
University of Pittsburgh Medical Center, Pittsburgh, PA.
Introduction: Next-generation sequencing (NGS) technology is emerging as a useful and potentially cost-effective tool in the clinical laboratory. A wide repertoire of bioinformatics applications exists for NGS data analysis; however, certain requirements of a clinical molecular laboratory limit their use; i) comprehensive report generation, ii) compatibility with existing laboratory information systems (LIS) and computer operating system, iii) knowledgebase development, iv) quality management (QM), and v) data security. Methods: SeqReporter, a web application based on client-server model, was developed using ASP.NET framework v4.0. The client-side was scripted using HTML5, CSS3 and Javascript. The server-side processing (VB.NET) relied on interaction with a customized SQL server 2008 R2 database. The database was comprised of a well-annotated in-house knowledge base and external variant databases (COSMIC, dbSNP, RefSeq, dbNSFP). Machine-learning algorithm implemented in SeqReporter facilitated enrichment of in-house knowledge base during routine signout based on pathologists’ decision of variant classification and annotation. Variant management and workflow documentation in the application’s database was designed to ensure compliance with CAP 2012 recommendations for NGS testing. Appropriate SSL/TLS network protocols were implemented for securing protected health information. Results: Overall, 104 tumors were sequenced on Ion Torrent PGM and primary analysis was performed by Torrent Suite v3.4.2. 1062 variant calls (VC) from 104 tumors were analyzed by SeqReporter. Each VC was classified into one of five report levels: i) known clinical significance, ii) uncertain clinical significance, iii) requiring pathologists’ review, iv) synonymous and deep intronic, and v) platform specific sequence errors. SeqReporter correctly annotated and classified 99.9% (859/860) of sequence variants, including 68.7% synonymous SNVs, 28.3% non-synonymous SNVs, 1.7% insertions, and 1.3% deletions. One variant of potential clinical significance was re-classified after pathologists’ review. Automatically-generated reports, subsequent to pathologists’ review, were compatible to our existing LIS. SeqReporter
also facilitated QM activities by monitoring variant trend and test order history. **Conclusions:**
SeqReporter is an example of a customized and well-designed informatics solution to optimize and automate the downstream analysis of clinical NGS data. It significantly minimized human introduced errors in variant classification and report drafting, therefore implementing efficient laboratory workflow and improving patient care. We propose it as a model, which may envisage the development of a comprehensive clinical informatics solution.