INTERVIEW

Tissue Preparation and Next-generation Sequencing Workflows for Oncology Testing

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INTERVIEW

Nahida Zaman: Molecular pathology is of increasing importance and a prerequisite for personalized cancer medicine.[1] The robust and standardized extraction of nucleic acids from formalin-fixed fixed, paraffin-embedded tissues for the analysis of predicted cancer mutations such as *KRAS*, *EGFR*, and *BRAF* is of special importance in routine molecular pathology.[2] Next-generation sequencing (NGS) has been described as arguably one of the most significant technological advances in the biological sciences in the last 30 years[3] and the cancer field is rapidly embracing this technology. Dr Ronald van Eijk and Dr Stein Crobach, you are both based within the Departments of Pathology of the Leiden University Medical Center in the Netherlands. Firstly doctors, welcome and can you please tell our readers a little bit about your background and the focus of your research, starting with Dr van Eijk?

Ronald van Eijk: I have been working in the Department of Pathology here in Leiden since 2001. Recently, I finished my thesis "Technological Advances in Molecular Pathology." I have been implementing a variety of new molecular techniques over the years and now we are working at implementing NGS technologies for molecular tumor diagnostics and research. We work under ISO comparable certification and our focus is on high-quality lab improvements by implementing automation and robotics.

Nahida Zaman: Thank you Dr van Eijk and over to you Dr Crobach.

Stijn Crobach: In 2009, I started my PhD project at the Department of Pathology. At the same time, I also started with my residency in pathology. What is important for us to see if we can get NGS into the clinics and to see what are the possibilities and the problems we see with introducing that technique. We are mainly interested in running this new technique on Formalin Fixed and Paraffin Embedded or FFPE material because that is the material we daily work with in the clinics.

Nahida Zaman: Thank you Dr Crobach. For a number of cancer subtypes, it is crucial to determine if the mutation is present in genes such as *KRAS*, *EGFR*, *BRAF*, and *PIK3CA* as test results will be consequently used to predict the response to therapy.[4] Your team and the Department of Pathology at the Leiden University Medical Center in the Netherlands have developed a remarkable

workflow process in the molecular tumor diagnostics group that results in tissue slides to first molecular analysis within 24 hours. Can you please describe this process and what were the major improvements versus your processes before this?

Ronald van Eijk: You refer to a video presentation we made last year and it's on http://www.scivee.tv/node/39348.[5] The start of the process is, from the standpoint of the pathologist, at the moment the tissue arrives in the lab, where it is prepared so as to be consulted by the pathologist. The tissue is sliced, stained, and is microscopically examined by the pathologist and it is decided if molecular testing is necessary for that tissue. Next micro-dissection is performed or tissue cores are taken. We start counting the analysis turn-overtime at the moment the samples are ready to load to our automated system for DNA and RNA isolation. After isolation, hydrolysis probe assays or TaqMan assays are performed which is a very fast process and a result will be available in 24 hours. If we cannot get a result from TaqMan then we will continue with Sanger sequencing or other technology to complete the molecular results. The whole process, from arriving of the sample material in the hospital, which can be sent in from other hospitals, until the moment that the actual result is going back to the clinics, that can vary from one to two weeks.

Nahida Zaman: Dr Crobach, what are the main challenges in implementing NGS workflows for oncology testing?

Stijn Crobach: NGS enables us to analyze many more genes than we did before and now we are in the process of validating the sequencing results. So getting data is not a problem to filter out the results of interest is a true challenge. This is difficult because you get enormous amount of data and you have to validate all data before you send these into the clinics. Another thing with NGS is that these techniques are changing so fast that the sequencer you would buy today is already out of date next year. So you have to keep up with the new developments. This makes standard workflows in clinics even more difficult.

Nahida Zaman: Dr Crobach, NGS is now maturing to the point where it is being considered by many laboratories for routine diagnostic use. Why is this?

Stijn Crobach: NGS enables to screen the complete genome in a very cost effective way.[6] In the past, you could analyze a few genes or part of genes. Now you can sequence the whole genome within one week and that offers enormous opportunities. The clinic also sees these opportunities and is asking more. With knowing more about changes that took place in the genome and you might predict response to therapy or prognosis based on the mutation profile you find in your genome. So there is a lot of information within the genome and now we're in the process of getting that information out and bring it to the patient.

Nahida Zaman: Dr Crobach, why have oncology applications specifically been such a natural bit for NGS data?

Stijn Crobach: Cancer is a disease of the genome. With NGS, you analyze the genome, so exposing the cause of the malignancies. Another practical thing is that in oncology most patients get surgery to be treated and in that way you get tumor material on which you can do research. So I think that is why NGS fits well into the oncology field. Now NGS is mainly focused on oncology, but the opportunities for using NGS are even broader.

Ronald van Eijk: Tumors proliferate over time so NGS can give you more insight into the development of these tumors over time. You can investigate primary tumors and also metastases. You can also study specific tumor populations and see how these behave over time. Due to treatment regimens the genetic consistence of the tumors can also change over time, and you can follow this process in much more detail with NGS.

Nahida Zaman: Can NGS data be trusted entirely or do you need confirmation by

standard routine methods such as Sanger or real-time PCR?

Ronald van Eijk: That is to be seen, currently what we and many other labs are validating the technology toward implementation in molecular tumor diagnostics. From a research standpoint, it may be acceptable to test a series of tumors and be allowed to miss something however in diagnostics you want to have a complete picture and you do not want to give erroneous results. Therefore, at this moment NGS results are compared to other technology like real-time PCR, digital PCR or Sanger sequencing. The first results are very promising.

Nahida Zaman: Dr van Eijk, when do you expect to introduce NGS on FFPE samples into your routine workflow and what kind of testing do you prefer, ie, panel, exome or whole genome?

Ronald van Eijk: We strive to implement the technology first quarter of 2014. There is still a lot of work to do in the validation of the assays and because the technology is evolving so fast it is difficult to tell at this moment what exactly will be implemented in the routine. We believe that the best way at this moment is to go for panel sequencing instead of whole exome or even whole genome sequencing. Of course this is a future plan but at this moment it seems better to start with a smaller panel in which we can tackle the genes having a direct implication for treatment or prognosis.

Steijn Crobach: Another issue that is also worth mentioning is that doing whole genome sequencing it is not a technical problem, but the enormous amount of data is the most problematic part. So, you also might be more pragmatic and test the genes within the genome that have consequences for the treatment of the patient.

Ronald van Eijk: A lot of this is to do with turnover time, with costs, and the decision to analyze the tumor and the normal material of the same patient. Now we can deliver results in 24 hours, for NGS that cannot be achieved yet. Anyway smaller gene panels can be easily analyzed on fast benchtop sequencers like the Myseq or PGM. This is a sharp contrast with HiSeqs or other high throughput sequencers where the turnover time will be more in the order of weeks instead of days.

Nahida Zaman: Dr van Eijk, the real importance of molecular pathology in clinical medicine has led to increased emphasis on optimized tissue preparation. How might some of the newer technologies aid pathologists?

Ronald van Eijk: For about 10 years or so we have been discussing what would be the best way to isolate DNA and RNA from FFPE material. Isolation is difficult because of the embedding process and degradation of the DNA in that and it is also very time-consuming and toxic chemicals like xylene are involved. Therefore, we have been searching for an automated system to do that whole process in one go. Robots are on the market for a while already but that first step of deparaffinization with xylene was never automated until recently. Now we have a system which can do that whole process in one go and that fitted quite well in our philosophy of having a high-quality laboratory with robotics automation. Now we do DNA isolation within four hours in a standardized way with very limited hands on time where operator-to-operator variation is excluded. Toward NGS in diagnostics, we believe that is a very important step in the workflow.

Stijn Crobach: And when you've got your DNA, you have to do your library preparation before you can sequence the DNA. Also for this process now robots are being developed. So, hands on time is decreasing every year. Thus, the whole process is step-by-step taken over by robots.

Nahida Zaman: Dr Crobach, recently relevant clinically biomarkers for certain cancer subtypes have been identified. Why is it important to decrease the turnover time in molecular analysis?

Stijn Crobach: Clinicians want trustworthy result so they can decide what kind of therapy they have to give to the patient. You do not want to wait for one or two weeks before you decide which treatment is optimal. It is beneficial for the patient to start as fast as possible with the most effective treatment. In this process, it is getting more and more important. From the moment that a patient might have cancer you want to treat the patients as fast as possible. Speed in the diagnosing and treatment process is also part of the quality. NGS that can produce a lot of data is a short-time fits in this process. So, NGS is not delaying the process of treating the patient.

Nahida Zaman: Dr van Eijk, why is the quality of extracted nucleic acid for applications like PCR, Sanger sequencing or next NGS so important?

Ronald van Eijk: There are a few remarks with what can be made. The problem in pathology is that the quality of the material is already reducing from the moment the tissue is taken from the patient in the operation room. Before it is embedded in paraffin, degradation can already take place, also in the process of isolation. So to a certain extent we do not have in our hands the quality of the material. Of course, if it arrives in the lab then we do not want to further degrade the material. This can be achieved with our automated method. We have seen a dramatic increase in the quality of our downstream applications for instance in Sanger sequencing. Another important issue to keep in mind in the analysis of DNA isolated from FFPE material is the type Taq polymerases you use. Many Taq polymerases are enhanced to improve the PCR quality, improve reading, etc., but many of these enzymes also need high-quality DNA to perform well which per definition is not the case for FFPE-DNA. Therefore, we searched for polymerases that do perform well on paraffin-extracted DNA.

Stijn Crobach: In case, your DNA of input is of less quality then also your analysis will be more difficult. The results you get are not as clear compared with high-quality DNA as input. In that sense also it is useful get the highest quality DNA as you can get because that makes the rest of the process and also the interpretation of the data easier. Many companies are aware of the degradation of DNA in FFPE material and they are also working on solutions that can work with shorter DNA fragments.

Nahida Zaman: What is the cost driver in the routine molecular pathology lab and how can you optimize this?

Ronald van Eijk: I believe you cannot speak of THE cost driver, it is probably not one thing, it is a combination. Cost for the employees, equipment and consumables, but also for data storage shouldn't be underestimated. Optimization can be achieved by implementing robotics or outsourcing of (parts of) the workflow. I think that one of the more difficult questions to answer is how to achieve a complete overview of the costs of the whole process, that is also part of the public discussion here in the Netherlands that you cannot really pinpoint what medical treatment costs.

Nahida Zaman: The analysis of NGS data remains a significant challenge. For experienced users, NGS data can be easily digested, however, the sheer scale of analysis, terminology and need for the command line, computer languages can be a formidable challenge. What procedures can laboratories implement to overcome this particular issue?

Ronald van Eijk: It is such a tremendous new technology but there is still so much in development on the scale of what you said in the question, analysis, terminology, computer languages, bioinformatics and we see that it is very hard to have all this in place in your own small laboratory, although we are very happy with the achievements in our lab. We see the need for more collaboration and in initiatives for well-equipped specialized centers with specialized employees so that we can keep pace with all the new developments. Develop-

ments are so fast that we are sure that in a few months, at the moment we have implemented NGS in our routine workflow, we have to decide to invest in the optimizing and validation of even newer and better technology to keep pace with the increasing demand of the clinic.

Stijn Crobach: What will be probably realistic in the near future is that there is one machine, in which you just put your patient sample, that will give you some genomic. The problem is that you are working with a black box. We think it is important that you know what is going on what are the problems you can expect. In that setting you need an experienced pathologist and molecular biologist and technicians that are familiar with the experiments, because of the evolutions are extremely fast in NGS, it is good to have all knowledge of the different parts of the process working closely together.

Nahida Zaman: What are the clinical implications of NGS for cancer medicine?

Stijn Crobach: This will have to be seen in the future but the expectations are huge. It used to be that cancers from the same organ were organized all in one group. For example, if you had cancer of your stomach that was one group of patients and stomach cancer patients all got the same treatment. Now what you see is that you are not treating the cancer based on where the cancer is located but you are more going to look to the genomic profile of the cancer and based on the genomic profile you decide which therapy someone will receive. This will change the way of treating patients and it will lead to a more effective way of treating patients. You can determine, before you start treating a patient, which therapy will probably be the most effective. Another thing that is interesting to look at if whether we can say something about prognosis of patients. Maybe certain mutation profiles will correlate with a bad prognosis studies will have to be performed in the coming years that shows to correlation between mutation status of specific genes and the outcome of those patients. There is a lot of work to be done but it will lead to personalized medicine. So at some point you are not treating the patient as a stomach cancer patient, but as a patient with stomach cancer specifically with that gene mutated, with this specific treatment and prognosis.

Nahida Zaman: Future demise on clinical data interpretation will be much greater both within the laboratory and in the clinic. Protocols for dealing with NGS data that guide what and how particular information will be reported and conveyed to the clinician will need to be established. How far away are we from the establishment of such guidelines?

Ronald van Eijk: Also in that direction, a lot of work has to be done. I was thinking of comparison with real-time PCR technology. PCR was invented in the 1980s but it lasted until 2009 until the first definitive guidelines which are called the MIQE guidelines[7] were put in place. There was a gap of about 20 years from the start of the technology until more or less definitive guidelines. At the other end, if you think of digital PCR[8] which is a very new development in real-time PCR, it only started five, six years ago, now already we see the first guidelines in place. We believe that also for NGS technology, although way more complex, in short time there will be very good checklists in place, analysis protocols, definitions about read-depth, definitions about quality of library preparations and guidelines for medical and ethical issues.

Stijn Crobach: To get those guidelines, collaborations centers with specialists of all the topics will help in getting these guidelines faster. In case, all hospitals in the Netherlands start doing NGS on their own it is more difficult to agree on guidelines and decide for example good thresholds settings in the analysis and other standardized methods.

Ronald van Eijk: Yes, there is an interesting discussion about what information coming out of NGS will be the most

useful to the clinicians. What role will the pathologist have in this. Will there be just a gene, mutation or expression list available for the particular tissue or patient or should direction to therapy be given? Anyway NGS technology will be implemented in many labs the coming years and efforts will be combined but only future will tell to what extend this technology will benefit the individual patient.

Nahida Zaman: Dr Ronald Van Eijk and Dr Stijn Crobach, thank you so much for taking the time to speak with us today, it has been a pleasure talking to you.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

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