Institute of Cell and Molecular Pathology
Director: Prof. Dr. med. B. Schlegelberger

Hannover Medical School

Moving on to the next generation – first experiences with the Roche GS Junior in *BRCA1/2* diagnostics

Kai Heinecke, Jessica Lohmann, Bernd Haermeyer, Bernd Auber, Nils von Neuhoff, Brigitte Schlegelberger, Doris Steinemann

Institute of Cell and Molecular Pathology, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany

1) Introduction

During the last few years, application of massively parallel sequencing in diagnostics increased dramatically[1]. The robustness of the technique has been shown[2] with benefits of next generation sequencing including:

- analysis of multiple patients and/or multiple loci per patient per run
- the ability to find mutations in small subclones due to a high sequencing coverage
- decreased analysis costs

Considering these advantages, we are planning to use the NGS system for
1) the genetic analysis of hereditary cancers, and
2) the genetic analysis of leukemias.

NGS should be able to speed up the analysis of known cancer genes (e.g. *BRCA1/2*) which is done at the moment by pre-screening (HRM, DHPLC) and subsequent Sanger sequencing, and to analyze small leukemic subclones for MRD diagnostics or mutation detection. We decided to start with the *BRCA1/2* diagnostic, as this method is already well-supported.

2) Multiplicom *BRCA1/2* MASTR assay

The MASTR assay from Multiplicom (Niel, Belgium) is widely used for *BRCA1/2* analysis. Using this approach, it was possible to adopt some of the established workflows, as the amplicon-based kit does not differ much from the techniques used in Sanger sequencing.

93 amplicons representing all coding regions of *BRCA1* and *BRCA2* are generated in five multiplex PCR-reactions per patient. Sample-specific molecular barcodes (MIDs) and Roche sequencing adaptors are incorporated via universal tags in a second PCR. The output can be pooled and directly used for the following clonal amplification for NGS.

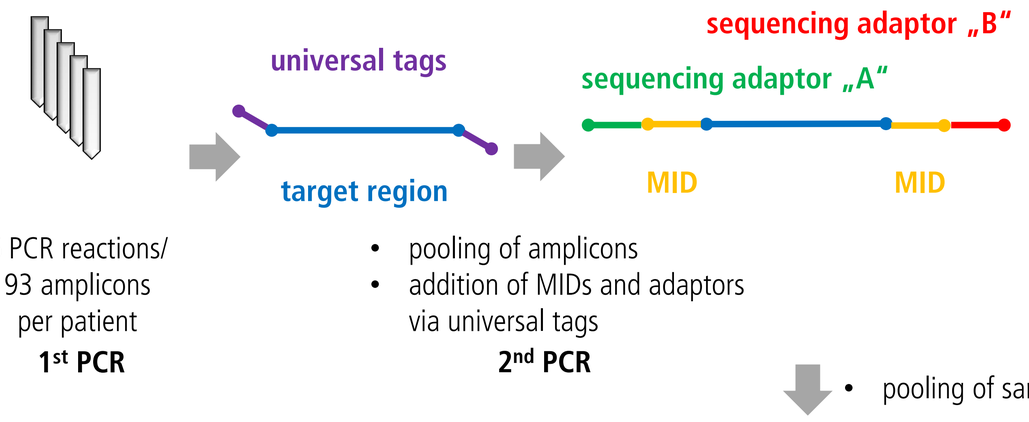

Fig. 1: Workflow of the Multiplicom MASTR assay.

3) The Roche/454 GS Junior system

The Roche/454 GS Junior is a Next Generation Sequencing system that uses the principle of pyrosequencing[3]. The guaranteed minimum output per run is 35 MBp and 70.000 high-quality reads. All runs we performed produced at least 45 MBb and 100.000 reads, so future runs will be calculated with these values. The best run achieved ~65 MBp and ~154.000 runs, which is a very good result for amplicon sequencing according to Roche.

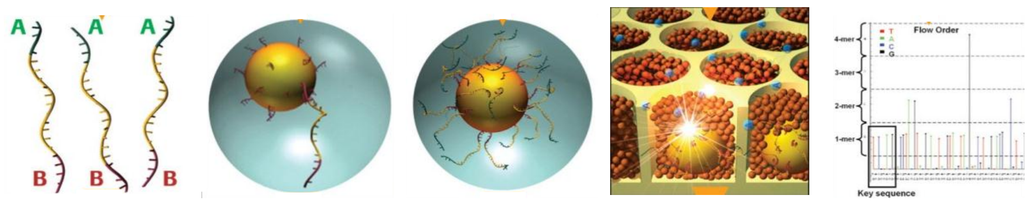

Fig. 2: Principle of 454 pyrosequencing. Left to right: amplicons derived from the second MASTR PCR can be directly used for clonal amplification on beads in an emulsion PCR (emPCR). The beads are loaded into a picotiter-plate, and the incorporation of nucleotides is monitored due to the activation of Luciferase. The result is a so-called flowgram, a graphic view of the number and type of incorporated bases per cycle (pictures provided by Roche).

Literature
[1] Desai AN et al., Clin Genet 2012 Jun; 81(6):503-10
[2] Kohlmann A et al., Leukemia 2011 Dec; 25(12):1840-8
[3] Metzker ML, Nat Rev Genetics 2010 Jan; 11(1):31-46

4) Analysis Software

We tested different software solutions for the analysis of the NGS datasets: the Amplicon Variant Analyzer (AVA) software provided by Roche, NextGene from Softgenetics (USA) and SeqNext from JSI Medisys (Germany). In the end, we decided to use SeqNext for routine diagnostics. The reasons were:

- Patient-based workflow
- Separated validation steps (technical/medical) and audit trail functions
- Flawless integration with the modules used for Sanger sequencing and MLPA

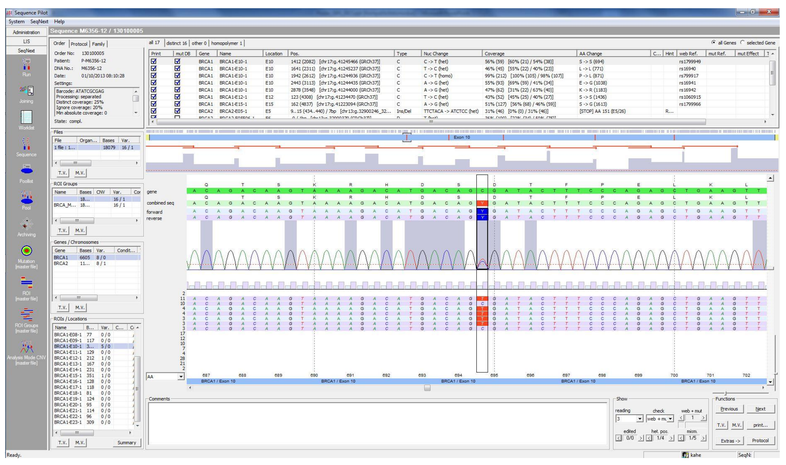

Fig. 3: User interface of SeqNext. Variations are shown in the upper panel. NGS data is presented as virtual peaks (middle panel), and consensus reads are shown in the lower panel.

5) Validation

We started validating the process by sequencing 13 samples with known variations in *BRCA1* or *BRCA2*. Different variation types are covered including a variety of known SNPs. All variations except for an exonic duplication could be detected with NGS. The detection of this large rearrangement was not possible due to low uniformity of the coverage between amplicons.

Gene	Variation (HGVS)	Variation (BIC)	Type	Detected
BRCA1	c.68_69delAG	c.187_188delAG	deletion	yes
BRCA1	c.213-12A>G	IVS5-12A>G	splicing	yes
BRCA1	c.564A>G	c.683A>G	same sense	yes
BRCA1	c.843_846delCTCA	c.962_965delCTCA	deletion	yes
BRCA1	dup Exon 13	dup Exon 13	exonic duplication	no
BRCA2	c.67+1G>C	IVS2+1G>C	splicing	yes
BRCA2	c.125A>G	c.353A>G	missense	yes
BRCA2	c.426-7_426-4delATTT	IVS4-7_IVS4-4delATTT	deletion	yes
BRCA2	c.426-12_426-8delGTTTT	IVS4-12_IVS4-8delGTTTT	deletion	yes
BRCA2	c.658_659delGT	c.886_887delGT	deletion	yes
BRCA2	c.3975_3978insTGCT	c.4206insTGCT	insertion	yes
BRCA2	c.9257-1G>C	IVS24-1G>C	splicing	yes
BRCA2	c.10095delCinsGAATTATATC	c.10322_10323delCinsGAATTATATC	indel	yes

In a second approach, samples from patients with unknown variation status were analyzed in parallel by classical Sanger sequencing and NGS. Results were identical, except for variations at the extreme ends of the amplicons that could not be detected due to different primer layouts.

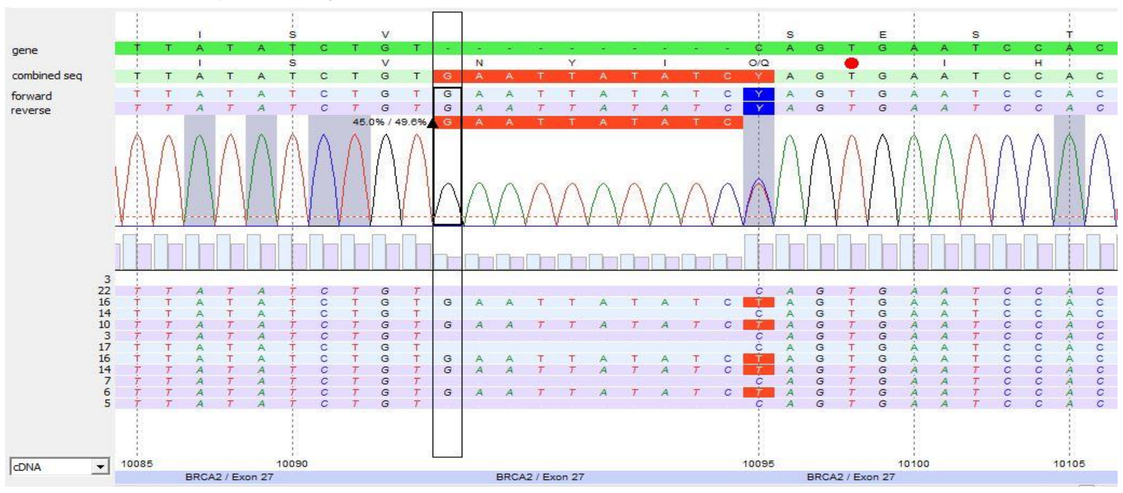

Fig. 4: The heterozygous indel c.10095delCinsGAATTATATC is easily recognizable in SeqNext.

6) Conclusion

Our experiences show that next generation sequencing is able to replace standard diagnostic techniques in *BRCA1/2* mutation detection. Especially for genes where certified kits are available and common workflows can be adopted, the switch to NGS is quite straight-forward. On the other hand, flexibility is limited if predesigned kits are used. However, large rearrangements like deletions and duplications cannot be detected yet so MLPA still has to be done in parallel.