Master's Thesis Engineering Technology

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Optimization and Validation of ddPCR Assays for the Detection of PIK3CA Hotspot Mutations in Liquid Biopsies of Patients With Invasive Breast Carcinoma

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Introduction and problem statement

Recently the efficacy of a drug called **alpelisib**, a treatment for terminal metastasized breast cancer patients with a ***PIK3CA* hotspot mutation**, is investigated in several clinical trials. The drug focusses on progression-free survival (PFS) and is administered to patients who already received several other treatments. To monitor the patient, a **liquid biopsy**, in this case blood, has to be taken and analyzed. Because the concentration of cell-free tumoral DNA (ctDNA) isolated from plasma is limited in comparison with the concentration of DNA obtained using tissue samples, a very sensitive detection method has to be used such as ddPCR (Bio-Rad). Therefore, the **optimization and validation** of the *PIK3CA* **ddPCR technique** is the main goal of this work. The second goal is to perform cell-free DNA (cfDNA) extractions using two different extraction methods. The method that yields the highest cfDNA concentration will be implemented in the routine.

Droplet digital PCR (ddPCR) principle

The principle of operation for the ddPCR technique is similar to the standard PCR however the embodiment is fundamentally different. Unlike the standard PCR, the reaction mixture is not put into a single well of a 96 well plate but in many small **sample-oil droplets**. These droplets are transferred to one single well whereby **thousands** (max 20,000) **of individual reactions** will take place in one well. The chance of amplification with this technique is much higher compared to the standard PCR technique whereby a **higher sensitivity** can be achieved [1]. After amplification, the droplets are screened whereby a 2-D scatterplot is generated as shown in **Figure 1**. The droplets are clustered into four groups and colors:

- The black cluster are double negative droplets that do not contain any DNA;
- The green cluster are HEX positive droplets that contain only wildtype DNA;
- The blue cluster are FAM positive droplets that contain only mutated DNA;
- And the orange cluster are double positive droplets that contain both mutated and wildtype DNA [1].

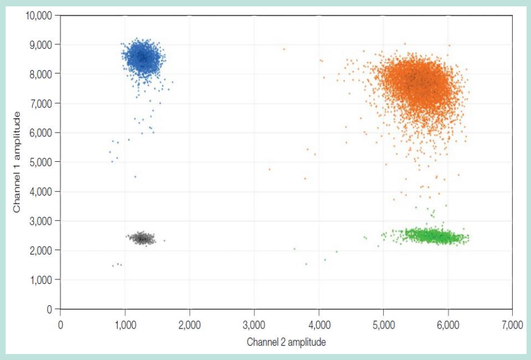

Figure 1 Example of a 2D-scatterplot

Material and methods

cfDNA plasma extractions
- Cobas® cfDNA Sample Preparation Kit (Roche)
- Maxwell® RSC LV ccfDNA Kit, Custom (Promega)

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***PIK3CA* ddPCR assay optimization**
- Testing validated Bio-Rad protocol with Maxwell extracts
- Adjusting amount of cfDNA input material from 8 µl to 6 µl

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***PIK3CA* ddPCR assay validation**
- Intra-run variation
- Inter-run variation
- Robustness
- Correctness

←

ddPCR mutation detection (20 patient samples)
- p.Glu542Lys
- p.Glu545Lys
- p.His1047Leu
- p.His1047Arg

Results

Cobas VS Maxwell plasma extractions

The results from **Figure 2** indicate that:
- 2 ml plasma available: Cobas extraction is preferred;
- 4 ml plasma available: Maxwell 4 ml extraction is preferred (capability to obtain a measurable result).

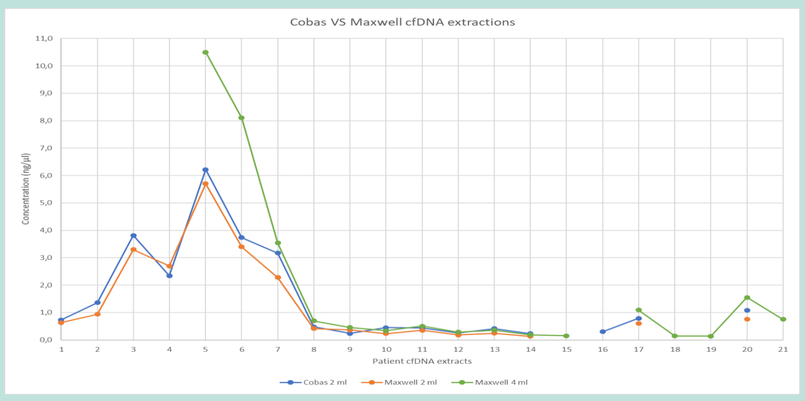

Figure 2 Comparison of the 3 different extraction methods

PIK3CA ddPCR assay optimization

- No inhibitory effects are observed when using Maxwell extracts;
- The validated Bio-Rad® protocol can be performed using 6 µl input material.

PIK3CA ddPCR assay validation

Intra-run variation ✓	Inter-run variation ✓	Robustness ✓
• Concordance between succesive duplo results	• Concordance between expected results VS experimental results	• Concordance between two executors

ddPCR mutation detection

- Tested on 20 patients with metastatic breast cancer (12 negative and 8 positive);
- Discordance for 2 of 20 patients;
- Additional ddPCR experiments performed using tumor tissue DNA, results are shown in **Table 1**.

Table 1 Discordance NGS result VS ddPCR plasma result patient 3 & 4

	Patient 3		
	Tissue NGS	Additional tissue ddPCR	Initial plasma ddPCR
Glu542Lys	-	+	+
Glu545Lys	-	-	+
His1047Leu	-	+	-
His1047Arg	+	+	+
	Patient 4		
	Tissue NGS	Additional tissue ddPCR	Initial plasma ddPCR
Glu542Lys	-	+	+
Glu545Lys	+	+	-
His1047Leu	-	Not performed	-
His1047Arg	-	Not performed	-

Hypotheses for the obtained results are:

- Tumor heterogeneity
- Lower sensitivity of NGS on tissue in comparison with ddPCR

Conclusion

The use of the **4 ml Maxwell extraction method is preferred** because it generates the highest concentration yields and obtains measurable results for low concentration samples while the other extraction methods can't. The optimization of the *PIK3CA* ddPCR shows that the **validated Bio-Rad protocol can be performed using 6 µl of cfDNA** whereby the assays shows **no inhibition** when using Maxwell cfDNA extracts. The **validation** of the PIK3CA ddPCR assays is performed by examining the intra-run variation, inter-run variation and robustness using positive and negative controls. The experimental results **met all of the predefined criteria** which shows the ddPCR assays can be used to detect the *PIK3CA* hotspot mutations. A **concordant result** between tissue and plasma is observed for **18 of 20 patient samples** whereby one sample, with only a His1047Arg mutation on tissue, showed Glu542Lys, Glu545Lys and His1047Arg mutations in plasma. In the other sample, Glu545Lys was detected on tissue and Glu542Lys in plasma. The sensitivity and specificity are 87.5% and 95.8% respectively. A good correlation is observed between the detection of *PIK3CA* mutations in tissue and plasma. The **validated assays will be included in the routine diagnostics** and provide an opportunity for the follow-up of the mutation status and for the support of the treatment strategy.

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[1] Bio-Rad, Writer, *Droplet Digital PCR - Rare Mutation Detection, Best Practices Guidelines (Bulletin 6628 Rev A).* [Performance]. Bio-Rad Laboratories, Inc.

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