GATCLIQUID: Analysis of circulating tumour DNA for cancer detection and monitoring

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Introduction

Recently, non-invasive analysis of circulating tumour DNA (ctDNA) and of circulating tumour cells (CTCs) from blood samples, the so-called liquid biopsy, has gained considerable attention. However, the description of circulating cell-free DNA is not entirely new and can be traced back to 1948. Already in these years and thus even before the double-helical model for the structure of DNA was proposed by Watson and Crick, Mandel and Métais described the appearance of extracellular nucleic acids in blood circulation[1]. It was not until the 1970s that researchers became interested in circulating cell-free DNA in the context of cancer and described an increase in the amount of DNA in the serum of cancer patients[2]. Yet, the cellular origin of this circulating cell-free DNA was unclear. In 1994, two groups independently reported tumour-associated mutations in plasma DNA of cancer patients and thus demonstrated that tumour cells can release DNA in the circulation[3,4]. Since then there has been growing interest in the detection and analysis of ctDNA – not only for the detection of cancer but also for monitoring therapy efficacy and for early identification of drug resistance.

Simultaneously, the analysis of circulating DNA has made an impact on many fields of clinical research including transplantation research[5], detection of viral DNA[6] and non-invasive prenatal testing for chromosomal aberrations[7,8]. Despite considerable worldwide interest in ctDNA, its detection has remained a major challenge for several reasons: 1) overall low levels of circulating DNA in most cases, 2) efficient discrimination between DNA from healthy and tumour cells and 3) an accurate quantification of mutated fragments present in a high background of wild-type fragments.

In the last years, GATC Biotech has worked intensively to overcome these difficulties in order to provide comprehensive solutions for the analysis of ctDNA. In 2012, GATC's affiliated company LifeCodexx successfully finalised its clinical study on non-invasive prenatal testing for trisomy 21 and was the first European company to employ this new, risk-free test method[9]. In addition, within publicly funded projects and with several clinical partners, GATC has extended its competences with circulating cell-free DNA to the field of oncology. With tens of thousands of DNA extractions from plasma followed by next-generation sequencing (NGS), GATC has gained strong expertise in circulating cell-free DNA analysis. This culminated in the release of the **GATC**LIQUID services including **ONCO**EXOME, **ONCO**PANEL and **ONCO**TARGET. Starting from only 4 mL of plasma and using validated workflows (Figure 1), GATC's ctDNA analysis can give answers to difficult scientific questions.

Excellent sensitivity and specificity allow **GATC**LIQUID to detect tumour DNA present in very low amounts. Compared to conventional tissue biopsy, liquid biopsy provides minimal discomfort and clinical risk to cancer patients. Analysis of ctDNA can address the problems of tumour accessibility and tumour heterogeneity, which frequently render tissue biopsies ineffective. As ctDNA often originates from resident dying tumour cells, it is possible to detect the tumour burden even before metastatic tumour cells have entered the blood-stream.

Here, we provide a detailed description of **GATC**LIQUID, including the workflows and the benefits of the appropriate analyses.

Isolation and characterisation of circulating cell-free DNA

The characteristics of cell-free DNA are quite different compared to genomic DNA from tissue and specialised protocols need to be followed for the extraction. Cell-free DNA is only present in very low amounts, is highly fragmented and contains little or no high molecular weight DNA. As DNA which is wrapped around nucleosomes is not accessible to nucleases, the DNA gets cleaved in-between the nucleosomes, leading to characteristic multimers of ~150 bp (Figure 2). This size distribution is reminiscent of the oligonucleosomal ladder of apoptotic cells[10].

As the concentration of cell-free DNA is very low, it is recommended to process at least 4 mL plasma (10 mL blood equivalent) to allow reliable downstream applications. Plasma from EDTA blood tubes should be prepared promptly within 1h after blood draw and frozen immediately to avoid any dilution of the cell-free DNA with DNA from lysed blood cells (Figure 3).

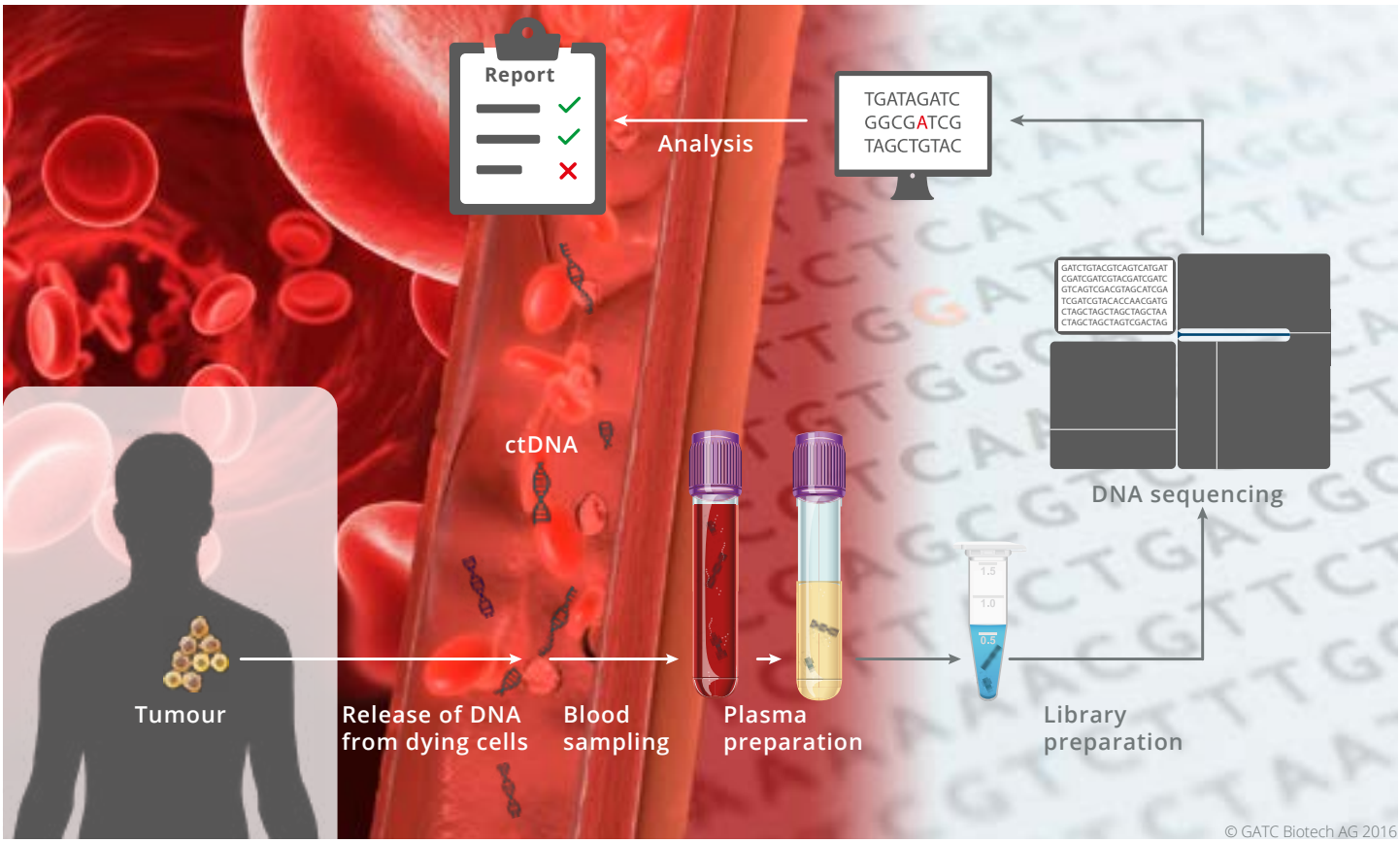

Figure 1: *Overview of ctDNA analysis exemplarily shown for* **GATC**LIQUID **ONCO**EXOME.

Recommendations for plasma preparation:

- ☐ Blood collection in EDTA or citrate BCT and plasma preparation within 1 h.
- ☐ Alternatively: blood collection in Cell-Free DNA BCT (e.g. Streck) and plasma preparation within 48 h.
- ☐ Standard centrifugation in swing out rotor.
- ☐ Additional centrifugation step to remove all cellular traces.
- ☐ Storage of plasma at -80 °C until DNA extraction.

Alternatively, specialised blood collection tubes can be used that stabilise cell-free DNA for several days[11]. In addition to plasma, serum could be used for the analysis of cell-free DNA, but is more susceptible to white blood cells lysis, leading to the dilution of cell-free DNA and decreased sensitivity. Therefore, GATC recommends the use of plasma over serum for cell-free DNA analysis. In order to maximise the quality of cell-free DNA, GATC has an optimised plasma preparation protocol with an additional centrifugation step to remove all cellular traces which already allowed successful preparation of thousands of samples. Short protocol of plasma preparation is shown on the left.

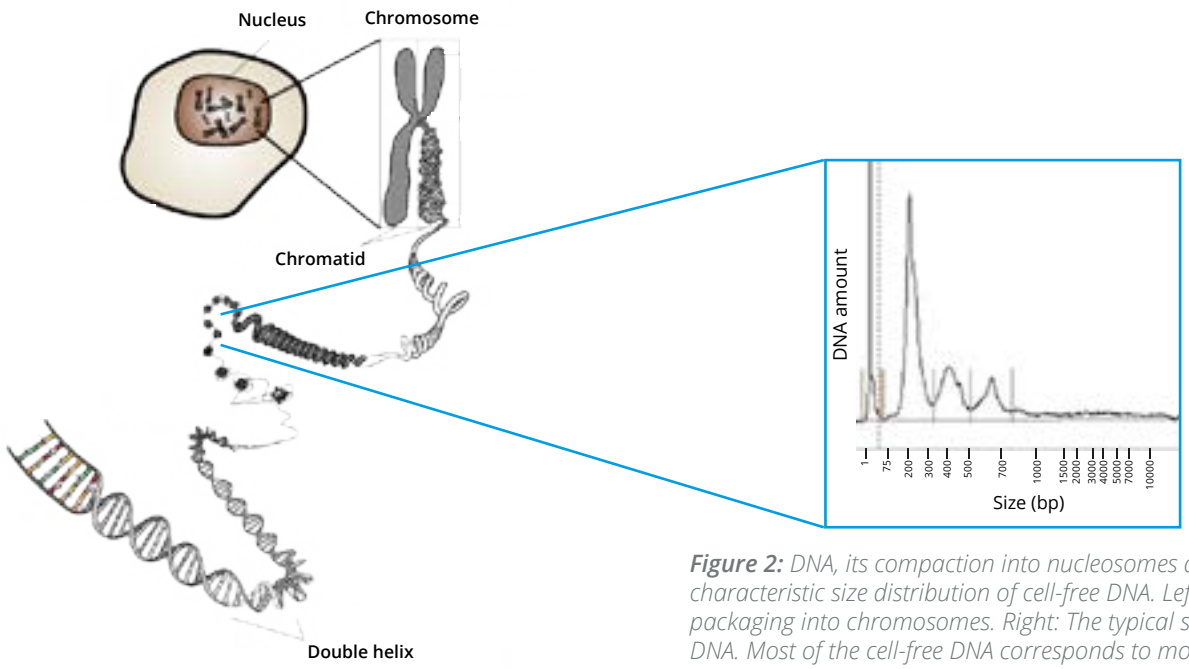

Figure 2: *DNA, its compaction into nucleosomes and the characteristic size distribution of cell-free DNA. Left: DNA and its packaging into chromosomes. Right: The typical spectrum of cell-free DNA. Most of the cell-free DNA corresponds to mono-nucleosomes with a size of ~150 bp and multimers of this size.*

ONCOEXOME - Unbiased overview of mutations in protein-coding regions

ONCOEXOME is a fully automated, high-throughput exome enrichment solution that enables researchers to discover somatic tumour mutations in ctDNA by next-generation sequencing. With sequencing of approximately 120 Mio read pairs, leading for the majority of samples to a coverage of 120-fold and more, **ONCO**EXOME reaches sensitivities down to 5 % and offers high performance in detecting mutations in cancer patients (Figure 4, Figure 5).

Due to the unbiased analysis of the whole coding region, it is possible to detect novel as well as known tumour mutations (e.g. mutations annotated in the COSMIC database) within the analysed ctDNA. Whereas tissue biopsies often only represent a small fraction of the tumour, the liquid biopsy-based **ONCO**EXOME analysis can provide a comprehensive picture of the genetic heterogeneity of the tumour. In Figure 4, we compared somatic mutations found in the tumour tissue of cancer patients with somatic mutations found by analysing the corresponding ctDNA of the same patients.

A high proportion of mutations are shared in tumour and ctDNA samples (23-45 %). In two entities (liver cancer and pancreatic cancer), even more mutations were found in the ctDNA than in the corresponding tumour mass of the patients. As shown in Figure 5, the mutation frequencies between tumour tissue and plasma ctDNA are highly correlated.

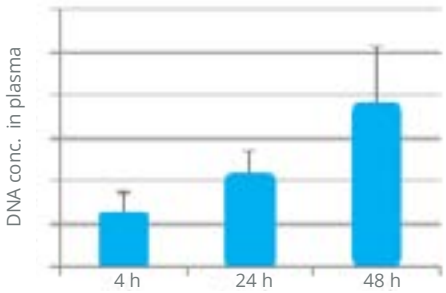

Figure 3: *Over time, the DNA concentration increases through contaminating DNA from lysed blood cells.*

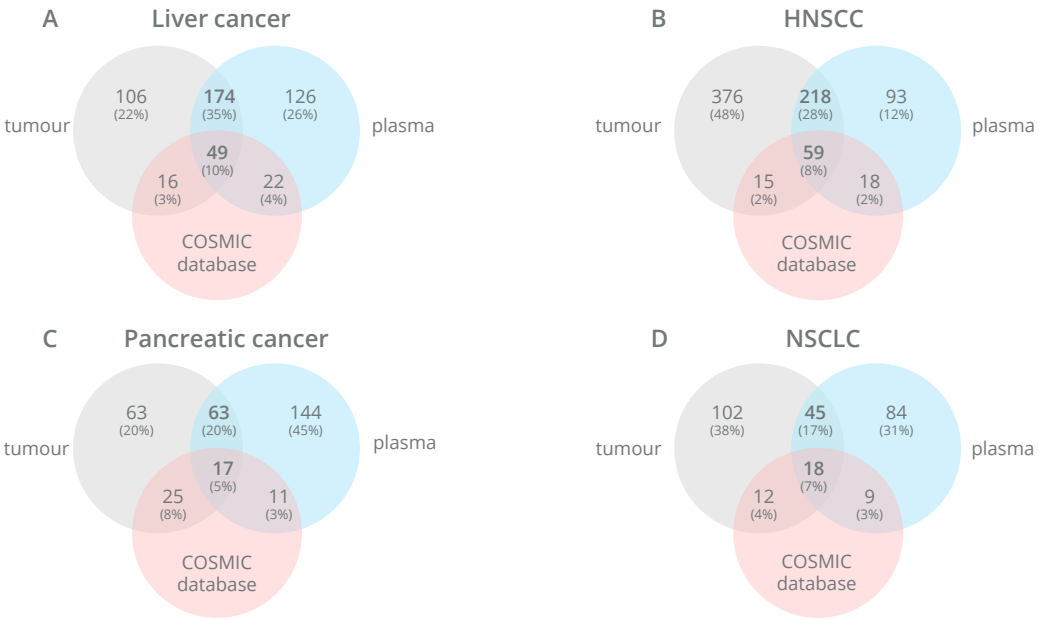

Figure 4: *Comparison of mutations found within the tumour mass versus mutations found in ctDNA. Venn diagrams showing the overlap between mutations found within the tumour mass and ctDNA in patients suffering from (A) liver cancer, (B) head and neck squamous cell carcinoma (HNSCC), (C) pancreatic cancer and (D) non-small cell lung cancer (NSCLC). To make sure that only tumour-relevant mutations are analysed, blood cells were investigated as 'healthy reference material' and the corresponding nucleotide variations were subtracted. Red circles highlight the proportion of detected mutations that are annotated in the COSMIC database (http://cancer.sanger.ac.uk/cosmic).*

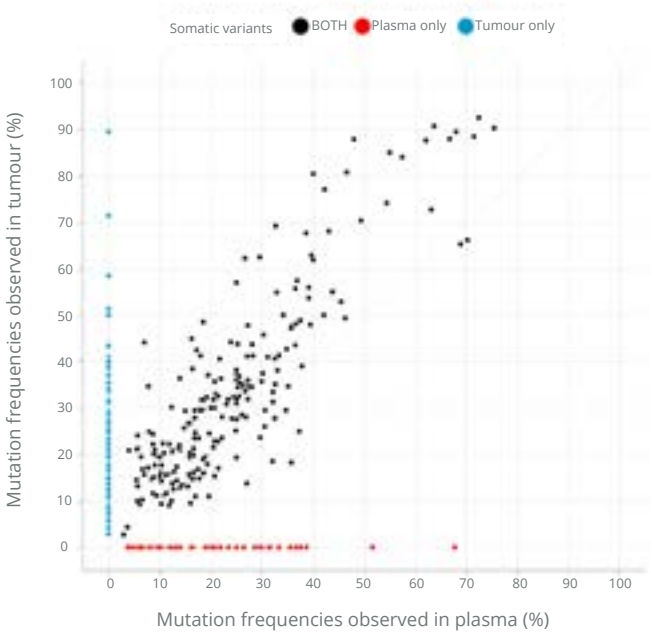

Figure 5: *Mutation frequencies of matched tumour tissue and plasma ctDNA. Shown is the mutation frequency which was detected within the tumour and within the plasma of a patient suffering from liver cancer. Most of the identified mutations are detectable in both fractions and are highly correlated.*

ONCOPANEL - Screening for key cancer drivers

ONCOPANEL is a comprehensive cancer panel for profiling important cancer mutations. Using single molecule PCR, which amplifies the DNA in millions of compartments, the sensitivity can be significantly increased compared to conventional PCR approaches. Optimised for very low amounts and highly fragmented DNA, this leads to an unbiased enrichment of a wide range of cancer-relevant targets, ensuring that tumour heterogeneity can be efficiently captured. The panel includes about 200 amplicons in 50 genes known to be associated with cancer, including the most important tumour suppressors and oncogenes (Table 1). Following single molecule amplification, the targets are sequenced using leading NGS technology with a minimum sequencing error rate.

ONCOPANEL achieves an excellent coverage of up to 10,000-fold (Figure 6) and a remarkable uniformity of more than 95 % at 0.2 x average coverage for all investigated targets (Figure 7).

In validation studies using a characterised mixture of DNA from different cell lines with known mutation frequencies, quantification with minor allele frequencies as low as 1 % with an excellent correlation (R^2 = 0.96) was achieved.

ONCOTARGET - Ultra-sensitive monitoring of cancer mutations

Gene Panel				
ABL1	EGFR	GNAQ	KRAS	PTPN11
AKT1	ERBB2	GNAS	MET	RB1
ALK	ERBB4	HNF1A	MLH1	RET
APC	EZH2	HRAS	MPL	SMAD4
ATM	FBXW7	IDH1	NOTCH1	SMARCB1
BRAF	FGFR1	IDH2	NPM1	SMO
CDH1	FGFR2	JAK2	NRAS	SRC
CDKN2A	FGFR3	JAK3	PDGFRA	STK11
CSF1R	FLT3	KDR	PIK3CA	TP53
CTNNB1	GNA11	KIT	PTEN	VHL

Table 1: List of 50 genes included in the **GATC**LIQUID **ONCO**PANEL.

ONCOTARGET allows ultra-sensitive detection of mutations of interest to monitor cancer patients during treatment or to detect a relapse at a very early stage. The underlying technology is droplet digital PCR which is considerably more sensitive and robust compared to real-time PCR[12]. Digital PCR was first invented by Sykes by partitioning a PCR reaction into small compartments so that each compartment only contains one '1' or no '0' template making the read out digital[13]. After end point amplification, each compartment is analysed for the presence of target fragments. Nowadays, the compartmentalisation is realised by an oil emulsification of the PCR mix resulting in a much higher number of compartments. Digital PCR quantifies the absolute number of templates and does not rely on standard curves[14]. Additionally, it is very robust against inhibitors and also minimises effects of non-optimal amplification efficiencies[12, 15].

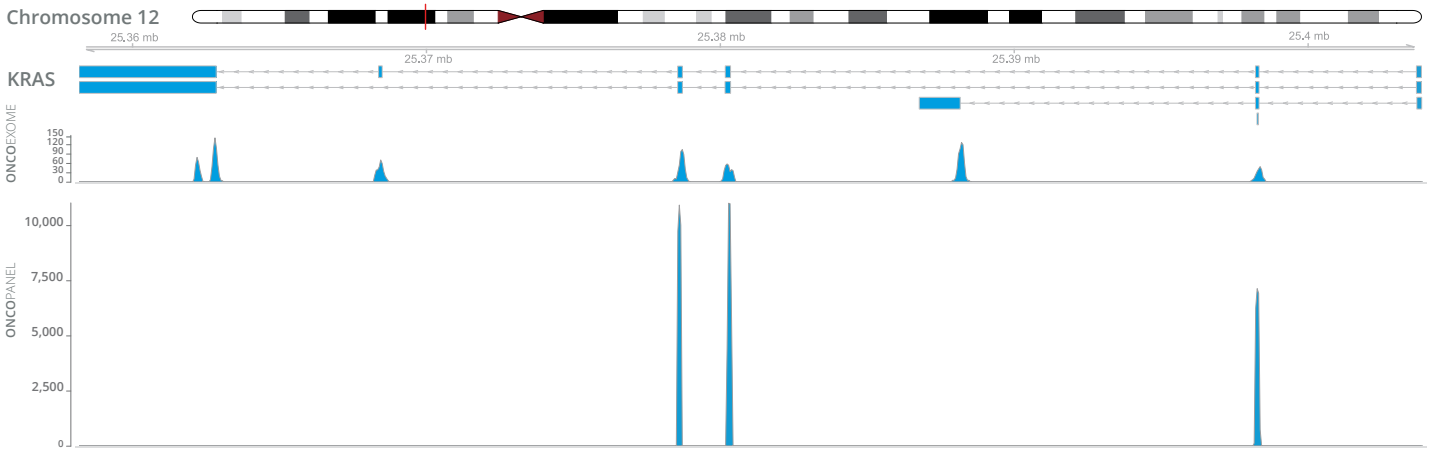

Figure 6: *Detection of minor allele frequencies using* **GATC**LIQUID *services.*
GATCLIQUID *analysis for parts of the KRAS oncogene.* **ONCO**EXOME *(upper panel) covers all coding regions and parts of the 5'-UTR of KRAS with a coverage of ~120-fold. With* **ONCO**PANEL *(lower panel), selected regions of the KRAS gene are analysed, leading to a coverage up to 10,000-fold.*

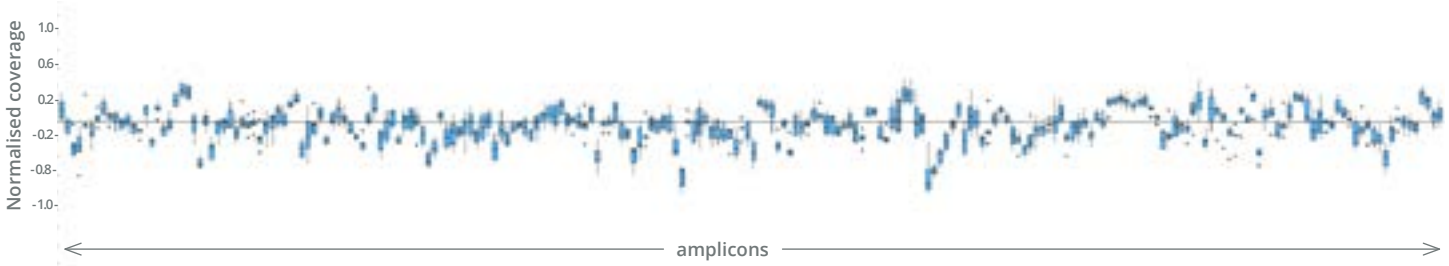

Figure 7: *Uniformity of amplified targets using the* **GATC**LIQUID **ONCO**PANEL.
Shown is the normalised coverage [log(amplicon coverage/panel coverage)] for all targets amplified with this cancer panel (n=6).

Currently, GATC offers assays for five common cancer mutations (Table 2). Here, performance with highest sensitivity and perfect correlation in dilution experiments is provided (Figure 8). Further assays for the detection of hotspot mutations within cancer relevant genes like NRAS, PDGFRA, TP53, GNAS, AKT1 and HER2, among others, are currently in development.

Gene	Mutation	Lowest observed frequency (%)	R^2
BRAF	c.1799T>A(V600E)	0.14	0.991
KRAS	c35G>A(G12D)	0.06	0.999
KRAS	c.38G>A(G13D)	0.16	0.997
PIK3CA	c.3140A>G(H1047R)	0.27	0.999
EGFR	c.2573T>G(L858R)	0.14	0.987

Table 2: *Mutations currently analysed with* **GATC**LIQUID **ONCO**TARGET.

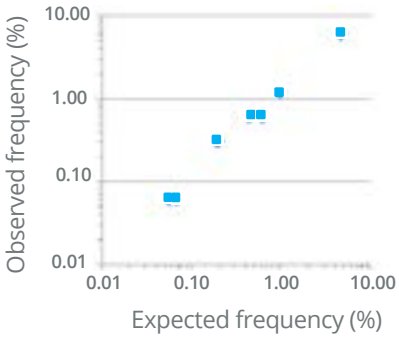

Figure 8: *Correlation between observed and expected frequencies using* **GATC**LIQUID **ONCO**TARGET. *Tumour samples with a well-characterised frequency of mutations were analysed. Even at below 0.1 % mutation frequency, there is an excellent correlation between observed and expected frequencies.*

Conclusion

The **GATC**LIQUID service family - including **ONCO**EXOME, **ONCO**PANEL and **ONCO**TARGET - allows for novel molecular insights into cancer biology (Table 3). The liquid biopsy-based services do not only enable early detection of tumours at stages where cancer cells have not yet spread, but the assays can also be used for a detailed molecular characterisation of a patient's tumour. Although the feasibility of liquid biopsy depends on tumour stage, blood vessel accessibility and tumour type, the assays remain a promising tool for non-invasive analysis of most solid tumours[16]. Undoubtedly, **GATC**LIQUID will facilitate the molecular characterisation of cancer patients, thus going one step further towards personalised medicine.

Contact

For more information please visit our website:
www.gatcliquid.com

Or if you have any questions, please contact us:
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Question / objective of the study	**GATC**LIQUID product
Comprehensive analysis of all exonic tumour mutations	**ONCO**EXOME
Detection of novel / patient-specific mutations in ctDNA	**ONCO**EXOME
Cancer profiling in cases where limited information about the tumour is available	**ONCO**EXOME, **ONCO**PANEL
Molecular stratification of tumours	**ONCO**EXOME, **ONCO**PANEL
Screening for well-known hotspots	**ONCO**PANEL, **ONCO**TARGET
Monitoring of cancer patients	**ONCO**PANEL, **ONCO**TARGET
Early detection of cancer relapse	**ONCO**TARGET

Table 3: *Range of scientific questions that can be addressed using* **GATC**LIQUID.

References

1 Mandel, P. & Metais, P., 1948, Les acides nucleiques du plasma sanguin chez l'homme. *C. R. Seances Soc. Biol. Fil.* 142: 241-243.

2 Leon, S. A., Shapiro, B., Sklaroff D. M. and Yaros, M. J., 1977, Free DNA in the serum of cancer patients and the effect of therapy. *Cancer Res.* 37: 646-650.

3 Sorenson, G. D., Pribish D. M., Valone F. H., Memoli V. A., Bzik D. J. and Yao S. L., 1994, Soluble normal and mutated DNA sequences from single-copy genes in human blood. *Cancer Epidemiol. Biomarkers Prev.* 3: 67-71.

4 Vasioukhin V., Anker P., Maurice P., Lyautey J., Lederrey C. and Stroun M., 1994, Point mutations of the N-ras gene in the blood plasma DNA of patients with myelodysplastic syndrome or acute myelogenous leukaemia. *Br. J. Haematol.* 86: 774-779.

5 Lo Y. M., Tein M. S., Pang C. C., Yeung C. K., Tong K. L. and Hjelm N.M., 1998, Presence of donor-specific DNA in plasma of kidney and liver-transplant recipients. *Lancet* (London, England) 351: 1329-1330.

6 Gan, Y. J., Sullivan, J. L. and Sixbey, J. W., 1994, Detection of cell free Epstein-Barr virus DNA in serum during acute infectious mononucleosis. *J. Infect. Dis.* 170: 436-439.

7 Lo Y. M., Patel P., Wainscoat J. S., Sampietro M., Gillmer M. D. and Fleming K. A., 1989, Prenatal sex determination by DNA amplification from maternal peripheral blood. *Lancet* (London, England) 2: 1363-1365.

8 Lo Y. M., Lo E. S., Watson N., Noakes L., Sargent I. L., Thilaganathan B. and Wainscoat J. S., 1996, Two-way cell traffic between mother and fetus: biologic and clinical implications. *Blood* 88: 4390-4395.

9 Stumm M., Entezami M., Haug K., Blank C., Wüstemann M., Schulze B., Raabe-Meyer G., Hempel M., Schelling M., Ostermayer E., Langer-Freitag S., Burkhardt T., Zimmermann R., Schleicher T., Weil B., Schöck U., Smerdka P., Grömminger S., Kumar Y. and Hofmann W., 2014, Diagnostic accuracy of random massively parallel sequencing for non-invasive prenatal detection of common autosomal aneuploidies: a collaborative study in Europe. *Prenat. Diagn.* 34: 185-191.

10 Jahr S., Hentze H., Englisch S., Hardt D., Fackelmayer F. O., Hesch R. D. and Knippers R., 2001, DNA fragments in the blood plasma of cancer patients: Quantitations and evidence for their origin from apoptotic and necrotic cells. *Cancer Res.* 61: 1659-1665.

11 Norton S. E., Luna K. K., Lechner J. M., Qin J. and Fernando M. R., 2013, A new blood collection device minimizes cellular DNA release during sample storage and shipping when compared to a standard device. *J. Clin. Lab. Anal.* 27: 305-311.

12 Hindson C. M., Chevillet J. R., Briggs H.A., Gallichotte E. N., Ruf I. K., Hindson B. J., Vessella R. L. and Tewari M., 2013, Absolute quantification by droplet digital PCR versus analog real-time PCR. *Nat. Methods* 10: 1003-1005.

13 Sykes P. J., Neoh S. H., Brisco M. J., Hughes E., Condon J. and Morley A. A., 1992, Quantitation of targets for PCR by use of limiting dilution. *Biotechniques* 13: 444-449.

14 Wittenberger T., Sleigh S., Reisel D., Zikan M., Wahl B., Alunni-Fabbroni M., Jones A., Evans I., Koch J., Paprotka T., Lempiäinen H., Rujan T., Rack B., Cibula D. and Widschwendter M., 2014, DNA methylation markers for early detection of women's cancer: promise and challenges. *Epigenomics* 6: 311-327.

15 Yang R., Paparini A., Monis P. and Ryan U., 2014, Comparison of next-generation droplet digital PCR (ddPCR) with quantitative PCR (qPCR) for enumeration of *Cryptosporidium oocysts* in faecal samples. *Int. J. Parasitol.* 44:, 1105-1113.

16 Bettegowda, C. et al., 2014, Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci. Transl. Med.* 6: 224ra24

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