Application Note

Unleash the Power of Liquid Biopsy: The GeneReader™ NGS System, now for circulating free DNA (cfDNA) at a 1% variant allele fraction

Farrah Winterbottom[1], Simon Hughes[1], Dietrich Lueerssen[1], John Blood[1], Hans Attig[2], Anja Schulz[2], Leif Schauser[3], Vikas Gupta[3], Sofie Jakobsen[3], Zara Ghazoui[1], Kate Bailey[2], Yi Kong[4], Tadd Lazarus[4], Thomas Rothmann[2]

[1] QIAGEN Manchester, UK
[2] QIAGEN GmbH, Hilden, Germany
[3] QIAGEN, Aarhus, Denmark
[4] QIAGEN Redwood City, CA, USA
Correspondence: Farrah.winterbottom@qiagen.com

QIAGEN® has been a pioneer in the liquid biopsy revolution. Our solutions empower laboratories to rapidly and accurately analyze circulating free DNA (cfDNA) to uncover valuable biomarkers for clinical cancer research.

The recent launch of the GeneReader Next Generation Sequencing (NGS) System offers the industry's first Sample to Insight® solution, removing the barriers to NGS adoption for any research laboratory. The GeneRead™ QIAact Actionable Insights Tumor Panel was designed specifically to enrich for the most relevant genes and variants, aimed at delivering actionable insights for cancer research[1]. The panel focuses on common cancer types that can benefit the most from genetic analysis: breast, ovarian, colorectal, lung cancers and melanoma. Previously we have shown that the panel, in combination with the GeneReader NGS System workflow, demonstrates consistently high performance with formalin fixed paraffin embedded (FFPE) sample material[1]. Here we present evidence that the same panel can also reliably detect variants from liquid biopsy samples containing tumor DNA fragments, at a 1% variant allele fraction threshold.

Introduction

To date, molecular characterization of cancer has primarily focused on FFPE biopsy samples. While FFPE-based sample analysis is well established, the approach is often limited by surgical feasibility, total tumor tissue availability and patient preference. Recent studies have revealed spatial and temporal heterogeneity of a tumor to be important factors in driving tumor behavior, including response or resistance to interventions, and suppression or recurrence of cancer thereafter[2-4]. Enabled by powerful NGS technology, a new field of study has emerged in recent years, with a focus on cfDNA, sometimes termed "Liquid Biopsy". These fragments of DNA are released by the tumor mass into the circulatory system, and mutational information from these can often shed light on tumor characteristics and disease progression. Liquid biopsy sampling enables detection of critical markers and allows real-time tracking of cancer molecular evolution through the course of the disease[3]. One such example is the EGFR gene, in which certain mutations have been identified to be the main cause for resistance development in multiple cancer types.[5,6]

While accepted as a potentially valuable source of information, liquid biopsy as a field is still nascent, and lacks integrated NGS workflow solutions with proven performance. QIAGEN is already an established expert in the field of sample preparation for liquid biopsy. Our specialized kits enable a laboratory to enrich and purify DNA from liquid biopsy samples of sufficient quantity and quality for NGS. The recently launched GeneReader NGS System is the industry's first true Sample to Insight workflow solution, thus facilitating NGS adoption for any research lab, including those lacking ample resources, specialized expertise or extensive NGS experience. With the addition of a liquid biopsy application for the GeneRead QIAact Actionable Insights Tumor Panel, we demonstrate a powerful tool to enable cfDNA cancer research through NGS.

Materials and Methods

Samples used for this study were commercially available cfDNA reference standards and commercially sourced clinical samples (human plasma).

Multiplex I cfDNA Reference Standards (Horizon®, cat. no. HD780) are cell line derived, fragmented human genomic DNA samples (representative of cfDNA samples) and contain clinically relevant variants present at known allelic frequencies that have been verified by digital droplet PCR (ddPCR)[7,8]. In this study, reference standards with allelic frequencies at or above 1% were used to confirm the performance of the GeneReader NGS System at detecting low frequency variants (typical of cfDNA samples). The DNA was quantified fluorometrically before use.

DNA was extracted from a set of 16 commercially sourced human plasma samples from patients with confirmed non-small cell lung cancer (NSCLC) using the QIAamp® Circulating Nucleic Acid Kit (QIAGEN, cat. no. 55114). DNA was eluted in 20 µl of elution buffer to ensure maximum DNA concentration. Extracted DNA was quantified fluorometrically. Extraction yields ranged from 0.4-2 ng/µl. The DNA was then analyzed using capillary electrophoresis to investigate DNA fragment size (cfDNA fragment size is approximately 160 bp).

DNA extractions from the Multiplex I cfDNA Reference Standards and plasma samples were run through the QIAGEN GeneReader® NGS System workflow (Figure. 1) according to the relevant handbooks (see Ordering Information and www.qiagen.com for full details of workflow components). The GeneRead QIAact Actionable Insights Tumor Panel was used for targeted PCR (QIAGEN, cat. no. 181910) and the GeneReader platform was used for next-generation sequencing.

A bioinformatics workflow specifically optimized for liquid biopsy applications on the GeneRead QIAact Actionable Insights Tumor Panel was developed using the QIAGEN Clinical Insight (QCI™) Analyze software to accurately identify variants down to a 1% allelic fraction. QCI Interpret was then used to assess actionability and provide clinical research insights on the variants identified.

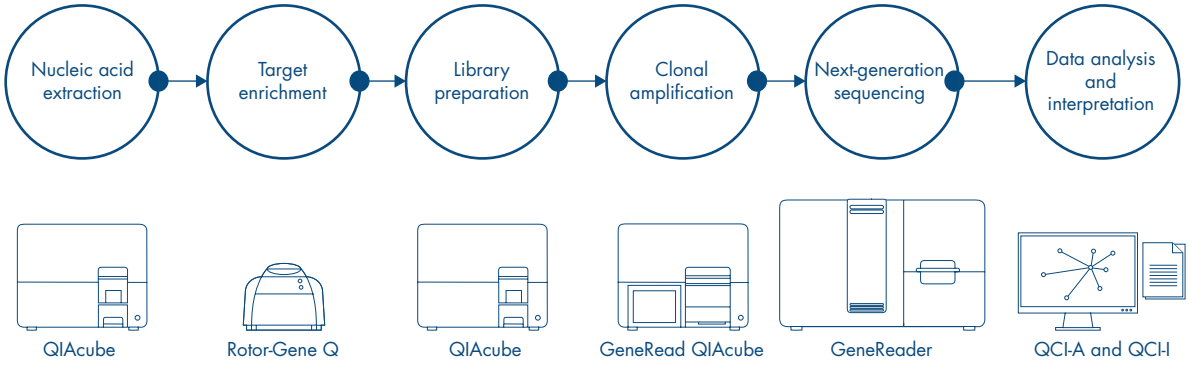

Figure. 1 The complete GeneReader NGS System workflow. Steps one to three (nucleic acid extraction to library preparation) may be performed either manually, or using one of QIAGEN's automation solutions, using the GeneRead Q family of products (see ordering information for details).

Results and Discussion

In order to investigate the ability of the GeneReader NGS System to detect variants at a 1% allelic frequency, we first tested the fully characterized Horizon cfDNA reference standards with the GeneRead QIAact Actionable Insights Tumor Panel. Not only was the workflow successful in correctly identifying all mutations covered by the panel, but observed allelic fractions were 100% consistent with those reported in ddPCR studies (Table. 1). Both single nucleotide variants (SNV) and insertion-deletion (INDEL) variants were identifiable at a 1% variant allele fraction threshold. Many of these variants, such as the EGFR L858R mutation, have proven to be relevant biomarkers for NSCLC[9, 10, 11].

Table. 1 Benchmark of the GeneReader NGS System liquid biopsy workflow on Horizon Multiplex I cfDNA Reference Standard material. Experimental data sets 1and 2 represent samples run at 4-plex and 6-plex respectively.

				GeneReader (GeneRead QIAact Actionable Insights Tumor Panel)			
Chr	**Gene**	**Variant**	**Expected Allelic Fraction (ddPCR Range)[8]**	**Experimental set 1 Observed frequency (Standard Deviation)**	**Average Coverage**	**Experimental set 2 Observed frequency (Standard Deviation)**	**Average Coverage**
7p12	EGFR	L858R	**1.00%** (0.6-1.4%)	**1.25%** (0.22%)	6.915	**1.29%** (0.29%)	6,156
7p12	EGFR	ΔE746 - A750*	**1.00%** (0.6-1.4%)	**1.01%** (0.29%)	10,340	**1.28%** (0.12%)	8,407
7p12	EGFR	T790M	**1.00%** (0.6-1.4%)	**0.89%** (0.38%)	1,226	**0.97%** (0.04%)	925
7p12	EGFR	V769 - D770insASV†	**1.00%** (0.6-1.4%)	**0.90%** (0.18%)	4,351	**0.86%** (0.01%)	4,004
12p12.1	KRAS	G12D	**1.30%** (0.78-1.82%)	**1.54%** (0.32%)	10,527	**1.32%** (0.39%)	9,884
1p13.2	NRAS	Q61K	**1.30%** (0.78-1.82%)	**1.57%** (0.21%)	11,757	**1.64%** (0.12%)	9,298
1p13.2	NRAS	A59T	**1.30%** (0.78-1.82%)	**1.41%** (0.15%)	10,552	**1.34%** (0.08%)	8,026
3q26.3	PIK3CA	E545K	**1.30%** (0.78-1.82%)	**1.23%** (0.25%)	36,384	**1.58%** (0.05%)	45,011

*ΔE746-A750 is an exon 19 deletion[10]
† V769 - D770insASV is an EGFR Exon 20 insertion[11]

A set of 16 human plasma liquid biopsies from cancer patients were then screened for EGFR-associated mutations using the GeneReader NGS System and GeneRead QIAact Actionable Insights Tumor Panel, in order to assess the performance of the system on biologically relevant human plasma samples. Of the 16 samples screened, seven samples were identified as EGFR mutation positive (L858R and a number of exon 19 deletions) (Table. 2). Moreover, these mutation calls were verifiable using PCR based technologies (e.g. the *therascreen*® EGFR Plasma RGQ PCR Kit (QIAGEN, cat. no. 870311).

These data show high performance consistency to be achievable with both cfDNA reference standards and plasma samples from patients with this workflow. This is the first study of its kind to systematically demonstrate the accuracy of a liquid biopsy assay combined with the robustness of a fully integrated NGS system.

It is important to note that the method of nucleic acid preparation method used and resulting sample quality have a major impact on the quality of the sequencing data. To support this process we suggest the following to ensure optimal results:

- Optimal extraction of cfDNA from liquid biopsy samples can be safeguarded by drawing blood into sample collection tubes that provide efficient stabilization of the liquid biopsy sample.
- Analysis of extracted DNA using capillary electrophoresis to check for contamination of high molecular weight material which may affect the performance of the sequencing system (e.g. with the QIAGEN QIAxcel® Advanced, cat. no. 9001941).
- Extraction of DNA from between 4-5 ml plasma and elution in the lowest stated volume in the QIAamp Circulating Nucleic Acid Kit handbook will help maximize DNA yields from liquid biopsy samples. Low DNA yields can present challenges such as providing insufficient input material for detecting low frequency variants. It is recommended that only samples with a DNA concentration of 1 ng/µl or higher should be used for NGS processing. Lower concentrations may be used, but this presents a risk to the outcome of the sequencing results.

Table 2. Performance of the GeneReader NGS System on commercially sourced human plasma samples (from oncology patients). Variant copies are calculated under the assumption that 1ng of cfDNA equals 360 genomic equivalents. Results for the Horizon Multiplex I cfDNA Reference Standards diluted to an input of 1 ng/µl are also included for variants ΔE746 - A750 & L858R for comparison

Sample	ng/µl input cfDNA	GeneRead QIAact Actionable Insights Tumor Panel Allelic Fraction Observed (%)	EGFR Variant	Variant copies
2072	0.84	2.8	ΔE746 - A750	34
2073	0.41	0.9	ΔE746 - A750	5
2077	0.45	1.3	ΔE746 - A750	8
2080	1.01	9.7	L858R	141
2081	0.7	1.3	ΔE746 - A750	13
2082	1.28	18.3	ΔE746 - A750	337
2083	2.12	5.7	ΔE746 - A750	174
Horizon cfDNA Reference Standard	~1	~1	ΔE746 - A750 & L858R	14

Conclusion

As our knowledge of tumorigenic mechanism and cancer evolution grows, there is an increasing desire and need to understand the real-time mutational landscape of tumors. Tracking the development of drug resistance using molecular markers has been shown to be a highly effective strategy for gaining a deeper understanding of the disease. Liquid biopsy has the potential to provide continuous monitoring of tumor evolution, where tissue biopsy is only able to deliver a single snap-shot in time. As such, the pairing of liquid biopsy with the right sequencing solution could help to unleash its full potential and offer a way to tap into this potentially invaluable pool of information.

The data presented here demonstrates the consistently high performance of a complete Sample to Insight NGS system with both liquid biopsy reference standards and biologically relevant plasma samples. Through combining QIAGEN's QIAamp Circulating Nucleic Acid Kit with the GeneReader NGS System and GeneRead QIAact Actionable Insights Tumor Panel, we were able to demonstrate reliable detection of critical cancer-relevant mutations at the 1% variant frequency threshold in the EGFR gene. Furthermore, these mutations were verified with a PCR–based method (e.g. *therascreen* EGFR Plasma RGQ). Using the workflow described, research laboratories can now analyze cfDNA samples accurately using a complete, single vendor NGS solution.

In summary, the GeneReader NGS System offers the following advantages for liquid biopsy:

- Unbiased, specific enrichment of high yield and quality cfDNA
- Verified pipeline tailored specifically for liquid biopsy sample analysis at high sensitivity and specificity
- High performance and confidence in sequencing results and interpretation

The GeneReader NGS System is the only fully integrated Sample to Insight NGS solution. Now with proven liquid biopsy data, it is well poised for any laboratory interested in cancer research.

References:

1. Oncology insights enabled by knowledge base-guided panel design and the seamless workflow of the GeneReader NGS System. Qiagen, 2015
2. Brouwer A, De Laere B, Peeters D, Peeters M, Salgado R, Diriz L, Can Leare S. Evaluation and consequences of heterogeneity in the circulating tumor cell component. Oncotarget. 2016; Epub ahead of print
3. Quin Z, Ljubimov VA, Zhou C, Tong Y, Liang J. Cell-free circulating tumor DNA in cancer. Chin J Cancer. 2016; 35:36
4. Dawson SJ, Tsui DW, Murtaza M, Biggs H, Rueda OM, Chin SF, et al. Analysis of circulating tumor DNA to monitor metastatic breast cancer. N Engl J Med. 2013;368(13):1199–1209.
5. Alix-Panabieres C, Pantel K. Clinical Applications of Circulating Tumor Cells and Circulating Tumor DNA as Liquid Biopsy. Cancer Discov. 2016; 6(5):479-91

6. Braig F, Voigtlaender M, Schieferdecker A, Busch CJ, Laban S, Grob T et al. Liquid biopsy monitoring uncovers acquired RAS-mediated resistance to cetuximab in a substantial proportion of patients with head and neck squamous cell carcinoma. Oncotarget. 2016; Epub ahead of print

7. Devonshire A, Fernandez-Gonzalez A, Whale A, Ng, SWJ, Amit H, et al. Independent study of Horizon Dx Cell Free DNA Reference Standards demonstrates strong concordance in the assessment of key material parameters. Horizon Discovery, Technical Note.

8. cfDNA Product Specification Sheet 6068 PSS-01 (V-01) for cfDNA Reference Standard Set, Horizon Discovery

9. Morrow CJ, Trapani F, Metcalf RL, Bertolini G, Hodgkinson CL, Khandelwal G et al. Tumorigenic non-small-cell lung cancer mesenchymal circulating tumor cells: a clinical case study. Ann Oncol. 2016; Epub ahead of print

10. Corominas-Faja B, Oliveras-Ferraros C, Cuyas E, Segura-Carretero A, Joven J et al. Stem cell-like ALDHbright cellular states in EGFR-mutant non-small cell lung cancer. Cell Cycle. 2013; 12(21):3390-3404

11. Oxnard GR, Lo P, Nishino M, Dahlberg S, Lindeman, NI, et al. Natural history and molecular characteristics of lung cancers harboring EGFR exon 20 insertions. J Thorac Oncol, 2013; 8(2):179-184

Ordering Information

Product	Contents	Cat. no.
GeneReader Platform	Next-generation sequencing instrument	9002312
QIAamp Circulating Nucleic Acid Kit	For 50 samples; concentration and purification of free-circulating DNA and RNA from plasma or serum	55114
GeneRead QIAact Actionable Insights Tumor Panel (GRTP-101X-12)	Wet-bench verified primer sets for targeted enrichment of tumor variants	181910
GeneRead DNA Library Q Kit	For 48 samples; 12 adapter primers for library multiplexing	185444
QIAcube 110V/230V	Robotic workstation for automation of Library Preparation	9001882/ 9001293
GeneRead Clonal Amp Q Kit	Clonal amplification of 4 library pools; automatable in the GeneRead QIAcube	185001
GeneRead QIAcube 110V/230V	Robotic workstation for automation of clonal amplification	9002344/ 9002345
GeneRead Sequencing Q Kit	Flow cell, sequencing buffers and sequencing add-ons	1853200
QIAGEN Clinical Insight (QCI) Analyze	Secondary analysis platform including Actionable Insights Tumor Panel Workflow	188001
QIAGEN Clinical Insight (QCI) Interpret	For NGS data interpretation and reporting	830371
QIAxcel Advanced	Capillary electrophoresis device	9001941
Multiplex I cfDNA Reference Standards	Standards representative of cfDNA samples, containing variants at know allelic frequencies	HD780 (Horizon)

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at **www.qiagen.com** or can be requested from QIAGEN Technical Services or your local distributor.

To learn more about the GeneReader NGS System, visit **GeneReaderNGS.com**

The GeneReader NGS System is for Research Use Only. Not intended for diagnostic procedures