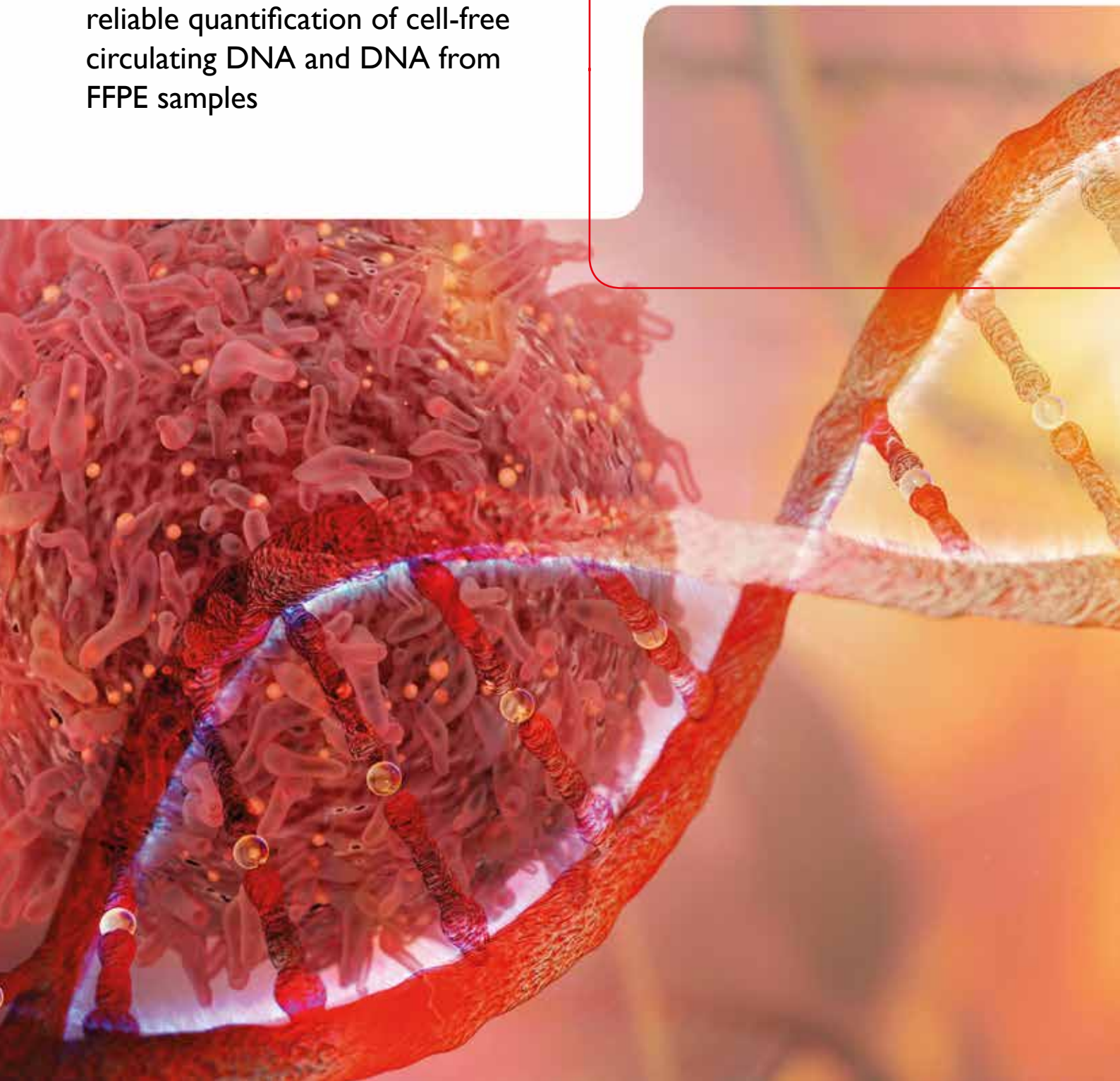


FROM LIQUID BIOPSY AND FFPE SAMPLES TO RESULTS

Fully automated purification and
reliable quantification of cell-free
circulating DNA and DNA from
FFPE samples



FULLY AUTOMATED PURIFICATION AND RELIABLE QUANTIFICATION OF CELL-FREE CIRCULATING DNA OR OF DNA FROM FFPE SAMPLES

Liquid biopsy is a new, minimally invasive technology for detection and analysis of biomarkers in blood or other body fluids without the need of invasive procedures. One important analyte is cell-free circulating DNA (cfDNA). The main challenges in working with cfDNA are the low concentration (1 - 50 ng/ml), including a fraction of up to 20% cancer DNA and the high degree of fragmentation of cfDNA (< 500 bp). Therefore processing higher sample volumes, typically in the range of several milliliters, is required. In order to address these obstacles STRATEC Molecular has developed a suite of products to

- Extract low amounts of fragmented DNA from 4 ml of plasma samples or from FFPE slides, fully automated on the InviGenius® PLUS instrument
- Reliably quantitate the extracted DNA using qPCR

These innovative solutions enable a reliable and reproducible application of cfDNA especially in the areas of oncology, extraction and analysis of fetal DNA from maternal plasma or improving the monitoring of transplant rejections. The individual products are designed to be combined in one efficient and robust workflow or as stand-alone process steps.

I. AUTOMATED EXTRACTION OF CIRCULATING CELL-FREE DNA

The InviMag® Free Circulating DNA Kit/ IG enables efficient, fully automated purification of free circulating DNA fragments from 4 ml of plasma or urine samples on the InviGenius® PLUS. The walk-away robotic system simplifies laboratory workflows and enables fully automated, standardized and highly efficient purification procedures.

BENEFITS

- Walk-away, highly reproducible and standardized cfDNA extraction from high sample volume (concentration from 4 ml down to e.g. 30 µl)
- Efficient recovery of short and fragmented high quality cfDNA
- Only 10 min hands-on time (manual kits about 60 min)

PRODUCT SPECIFICATIONS

Protocol:	Magnetic bead based isolation of cfDNA on the InviGenius® PLUS platform
Starting material:	4 ml of plasma, urine or plasma from stabilized blood samples, e.g. Cell-Free DNA BCT® tubes from Streck
Throughput:	1 - 12 samples per run
Yield of cfDNA:	15 - 150 ng from 4 ml plasma
Processing time:	2.5 - 3.5 h

a) REPRODUCIBILITY

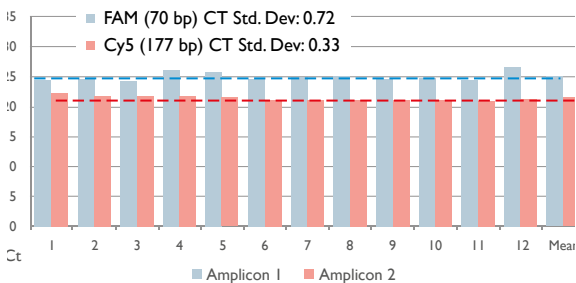


Fig. 1: Excellent and reproducible intra-run recovery of circulating cfDNA

Circulating cell-free DNA was isolated from 12 aliquotes of 4 ml Seracon plasma samples in parallel using the InviMag® Free Circulating DNA Kit/ IG on the InviGenius® PLUS and eluted in 100 µl. DNA yield was quantified by real-time PCR of two amplicons within the 18S rRNA sequence.

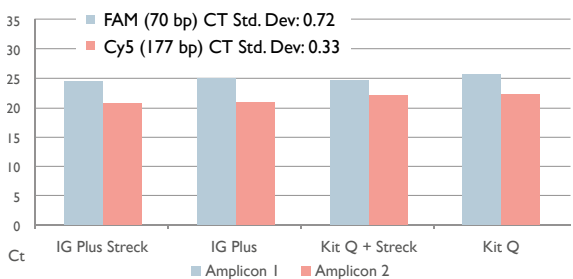


Fig. 2: Comparison of extraction efficiency using automated and manual kits

cfDNA was isolated from 4 ml plasma (same blood collected in Streck tubes and EDTA tubes) using the fully automated procedure of the InviMag® Free Circulating DNA Kit/ IG on the InviGenius® PLUS in comparison to a manual kit. DNA was quantified by real-time PCR of two amplicons within the 18S rRNA sequence (70 bp & 177 bp). Samples collected with Streck tubes yield comparable results to conventional EDTA tubes and the extraction efficiency is at least comparable to competition.

b) QUALITY OF PURIFIED THE cfDNA

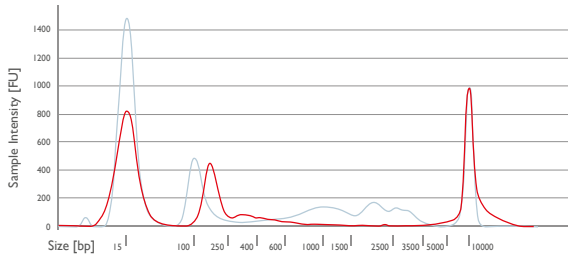


Fig. 3 Case study with 68 plasma samples - Fragment analysis on TapeStation

TapeStation 4200 electropherogram of InvimaMag[®] extracted cfDNA (red) compared with Q extracted cfDNA (blue) from 8 ml of plasma samples. Fragment analysis by TapeStation showed the characteristic cfDNA fragments of 170 bases in most of the extractions from both methods. Nevertheless some Q extractions showed additional signals of fragments above 500 bases.

Data kindly provided by Professor Jacqui Shaw and Dr Caroline Cowley, Leicester Cancer Research Centre, University of Leicester, UK

c) DOWNSTREAM APPLICATIONS OF THE PURIFIED cfDNA

Tab 1: Sequencing metrics show higher read depth for the InvimaMag[®] method in 3 of 4 samples

Sample Name	Gene ID	Allele Name	Variant	Total Read Coverage	Allele Read Coverage	Allele Read Frequency	Total Molecular Coverage	Allele Molecular Coverage	Allele Molecular Frequency
Competitor Q - A	TP53	p.M237I	T	210696	0	0	4283	0	0
InvimaMag [®] - A	TP53	p.M237I	T	192580	45	0.02	4154	3	0.07
Competitor Q - A	TP53	p.Y200C	C	94033	167	0.18	3052	5	0.16
InvimaMag [®] - A	TP53	p.Y200C	C	81237	101	0.12	2286	2	0.09
Competitor Q - B	TP53	p.R273C	A	76423	27	0.04	3706	2	0.05
InvimaMag [®] - B	TP53	p.R273C	A	116962	54	0.05	7388	4	0.05
Competitor Q - C	EGFR	p.E746_A750delELREA	-	159796	28013	17.53	2389	657	27.50
InvimaMag [®] - C	EGFR	p.E746_A750delELREA	-	122372	25809	21.09	6213	2025	32.59
Competitor Q - C	EGFR	p.T790M	T	76409	4359	5.71	1080	66	6.11
InvimaMag [®] - C	EGFR	p.T790M	T	120911	6261	5.18	5020	275	5.48
Competitor Q - C	TP53	p.R158L	A	36373	0	0	323	0	0
InvimaMag [®] - C	TP53	p.R158L	A	103963	115	0.11	2913	5	0.17
Competitor Q - D	BRAF	p.V600E	T	62493	43	0.07	1211	2	0.17
InvimaMag [®] - D	BRAF	p.V600E	T	15076	0	0.00	541	0	0
Competitor Q - D	TP53	p.R248W	A	97158	456	0.47	2065	7	0.34
InvimaMag [®] - D	TP53	p.R248W	A	180266	1417	0.79	7333	55	0.75

Plasma samples from different cancer patients (Breast Cancer, Non-Small Cell Lung Cancer, Small Cell Lung Cancer, Metastatic Melanoma) were used as starting material. cfDNA was isolated from 4 ml of plasma into an elution volume of 80 µl using the InvimaMag[®] Free Circulating DNA Kit/ IG on the InviGenius[®] PLUS in comparison to a manual kit Q. Targeted Next Generation Sequencing (OncoPrint cfDNA assay, ThermoFisher) showed comparable results (VAF) with both extraction methods.

Data kindly provided by Professor Jacqui Shaw and Dr Caroline Cowley, Leicester Cancer Research Centre, University of Leicester, UK

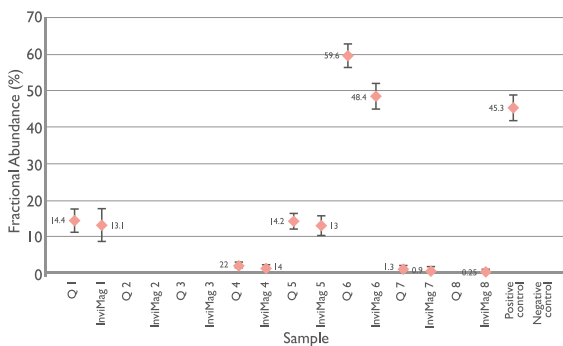


Fig. 4 Detection of ctDNA by ddPCR mutation analysis

BRAFV600K fractional abundance analysis of 8 samples
Circulating cell-free DNA was isolated from plasma samples of different cancer patients using the InvimaMag[®] Free Circulating DNA Kit/ IG on the InviGenius[®] PLUS in comparison to a manual kit Q. Digital droplet PCR (BioRad) was performed for:

BRAFV600K mutation (8 samples – Melanoma)
BRAFV600E mutation (28 samples – Melanoma)
EGFR T790M mutation (5 samples, NSCLC).

The ddPCR results were comparable with both extraction methods in terms of accepted droplet counts and fractional abundance of detected mutations. Both methods displayed 100% correlation for the detected mutations.

Data kindly provided by Professor Jacqui Shaw and Dr Caroline Cowley, Leicester Cancer Research Centre, University of Leicester, UK

2. STANDARDIZED & FULLY AUTOMATED EXTRACTION OF DNA FROM FFPE TISSUE

Genotyping tumor tissue in search of somatic genetic alterations for actionable information has become routine practice in research and clinical oncology. As FFPE as sample source for cancer testing becomes more widely adopted, laboratories seek to high quality solutions. The new InvimaMag[®] FFPE DNA Kit/ IG enables efficient, fully automated purification of genomic DNA fragments direct from single paraffin embedded tissue samples (1-5 FFPE slides) on the InviGenius[®] PLUS without any manual pretreatment.

BENEFITS

- Fully automated extraction of DNA from FFPE samples - high reproducibility through standardized processing
- Efficient recovery of short and fragmented ready-to-use DNA
- No manual pretreatment like deparaffination or formalin removal required
- Automated method saves 60 % of hands-on time
- 15 min hands-on time

PRODUCT SPECIFICATIONS

Starting material: max. 3 sections, thickness of 10 µm + surface area up to 200 mm² each
or max. 6 mm³ in volume
or 6 mg in weight for paraffin + sample

Protocol: Magnetic bead based kit for DNA isolation, for use in combination with the InviGenius[®] PLUS
Throughput: 1 - 12 samples per run
Elution Volume: 80 µl input; 50 µl output
Yield: 2 – 200 ng (depending on sample)
Processing time: 3.5 - 4 h (including 2.5 h lysis time)

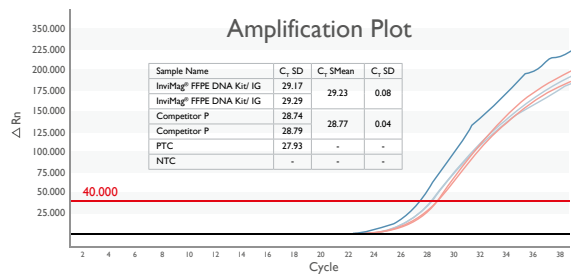


Fig. 1 Comparable results using different manual and automated isolation methods

DNA was isolated from one FFPE slide of a primary tumor (duplet) using a manual kit from competitor P (blue curves) and fully automated using the InviMag[®] FFPE DNA Kit / IG on the InviGenius[®] PLUS (pink curves). The extracted DNA was analyzed in a K-ras specific real-time PCR. The amplification plot shows comparable CT values for both extraction methods (measured in duplicates).

Pink: InviMag[®] FFPE DNA Kit / IG (prototype)
Green: Positive control
Blue: Competitor P

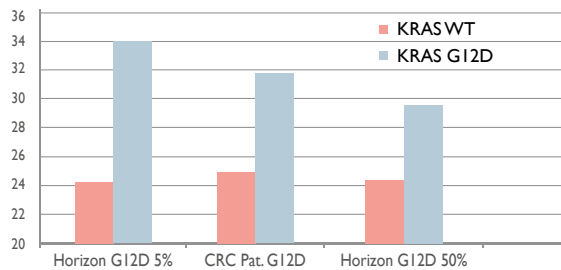


Fig.2 KRAS mutation detection in a patient sample

DNA extracted fully automated from FFPE sections using the InviMag[®] FFPE DNA Kit / IG on InviGenius[®] PLUS were subjected to the InviGene[®] KRAS 12/13 Kit defined FFPE sections (Horizon Discovery) with an allelic frequency of 5 and 50% KRAS G12D.

- defined FFPE sections (Horizon Discovery) with an allelic frequency of 5 % KRAS G12D
- FFPE section with a KRAS G12D mutation (confirmed by Sanger sequencing) from a colorectal cancer (CRC) patient, lying between 50% and 5% due to the detectable mutation rates from 5 - 50% by Sanger Sequencing
- defined FFPE sections (Horizon Discovery) with an allelic frequency of 50 % KRAS G12D

COMPARISON OF FULLY AUTOMATED VERSUS MANUAL EXTRACTION METHOD

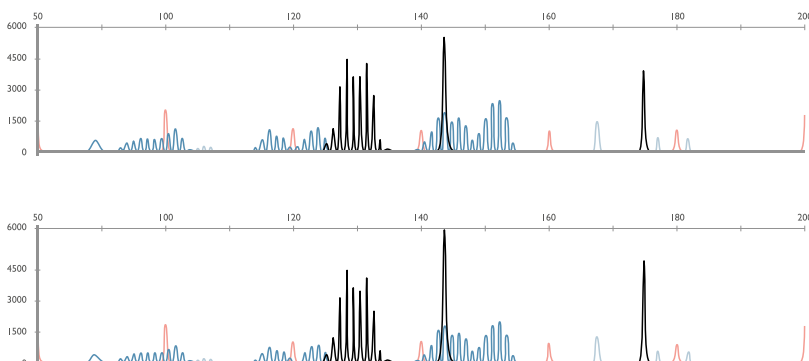


Fig.3 MSI Assay Analysis

Electropherogram of colon cancer sample 2, the upper panel is the manual extraction while the lower panel is the fully automated extraction.

For 8 diagnostic samples (4 slides each sample, 5 µm each slide) including colon cancer, lung cancer and melanoma, DNA was extracted from 4 or 8 FFPE tissue sections using a manual kit and the fully automated **InviMag® FFPE DNA Kit /IG** on the **InviGenius® PLUS** platform. DNA yield, purity and quality were determined using the Quant-iT™ PicoGreen® DNA Kit on the Fluoroskan Ascent (WI-81), Nanodrop ND-1000 (EU-47) and Agilent DNA Chip Analysis on the Agilent 2100 Bioanalyzer (WI-90).

Tab 1. The table shows the DNA yield in ng measured by Picogreen and the DNA purity measured by Nanodrop in comparison of automated and manual extraction methods.

Tumor type	Picogreen DNA yield (ng)			DNA purity (A260/280)		
	manual (M)	Walk-away (IG)	IG versus M	manual (M)	Walk-away (IG)	IG versus M
Colon 1	3.03	10.67	351.9	1.75	1.84	105.1
Colon 2	7.46	14.61	195.8	1.90	1.90	99.7
Lung 1	9.89	11.28	114.1	1.83	1.82	99.2
Lung 2	3.75	6.02	160.4	1.77	1.88	106.2
Lung 3	19.66	54.36	276.5	1.91	1.85	96.8
Lung 4	13.37	15.01	112.3	1.90	1.81	95
Melanoma 1	10.99	45.11	410.4	1.86	1.92	102.9
Melanoma 2	15.09	14.85	98.4	1.85	1.98	106.7

Data kindly provided from Histogenex, Belgium

There is no significant difference between the DNA yield generated with the fully automated extraction on the InviGenius® PLUS and the manual kit, but across all samples, the amount of dsDNA obtained with the fully automated extraction was more than 80% of the amount of dsDNA obtained with the manual extraction procedure (average: 162.48%). Furthermore, there is no significant difference in DNA purity and DNA quality between the two extraction methods. All extracts are suitable for MSI testing and Cobas mutation testing. Both extraction methods show comparable results when performing extraction from medium size or large FFPE tissue sections.

3. QUANTIFICATION OF DNA PRIOR TO COST INTENSIVE DOWNSTREAM APPLICATIONS

The InviQuant GeneCount 40 is a qPCR-based system to determine the quantity of human genomic DNA e.g. prior to use in NGS or other downstream applications. Unlike other approaches that target a single genomic locus, the InviQuant GeneCount 40 uses a qPCR assay to detect 40 genomic loci which are randomly distributed throughout the human genome. The proprietary design ensures minimal variation caused by local genomic events. The use of the included high-quality DNA standard allows quantification of amplifiable targets in cfDNA samples.

Internal and external studies suggest that results for the quantification of cfDNA from plasma samples between the Qubit / Bionalyzer and the quantitative PCR do not match in many cases. Differences of 50 to even 100% between the methods are fairly common. Since the qPCR assay directly measures the amplifiable portion of DNA it is the method of choice to analyze this specific sample type.

BENEFITS

- Provides qPCR based DNA quantification of up to 200 samples
- Quantifies isolated cfDNA or traces of genomic DNA that is amplifiable by PCR, suitable for determining the correct amount of input DNA for NGS application
- Increased sensitivity in comparison to single copy qPCR through detection of 40 randomly distributed genomic loci
- Method of choice for cfDNA quantification, qPCR assay directly measures the amplifiable portion of DNA

PRODUCT SPECIFICATIONS

Processing volume: 25 µl (5 µl DNA)
 gDNA input concentration: between 10 pg/µl to 2.5 µg/µl
 Processing Time: 60 min (preparation and cycling)
 Standard for quantification: 100.000 PCR product copies
 Storage: at -20°C

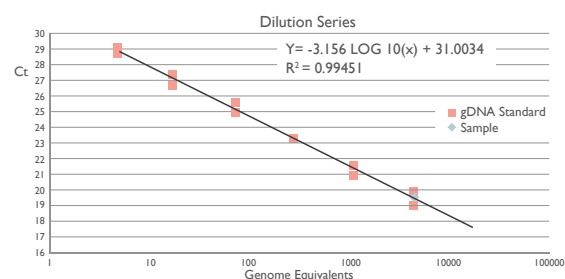


Fig. 1: Dilution series

Fig. 1 Genomic DNA with known quantities of genome equivalents (6 pg $\hat{=}$ 1 diploid genome) was serially diluted. Serial dilutions were measured by qPCR using the **InviQuant GeneCount 40**. Ct-values were plotted against the known genome equivalents to generate a standard curve and its linear equation. All samples were measured in duplicates. Genome equivalents for the sample (blue) were calculated using the linear equation.

$$E.g. 10^{(31.0034 - 19.5) / 3.156} = 4894 \text{ genome equivalents}$$

INVIGENIUS® PLUS

The InviGenius® PLUS is a true walk-away system for DNA/RNA extraction and purification from clinical samples – providing a reliable “Sample in – Eluate out” technology! The combination of well-established magnetic bead based InviMag® technology and state-of-the-art process automation allows for standardization and streamlining of laboratory workflows. Innovative functionality and optimized protocols for demanding samples and applications result in reliable performance and superior DNA and RNA quality for molecular diagnostics*.

BENEFITS

- Extract and purify DNA and RNA from up to 12 liquid samples in parallel
- Direct processing from primary tubes
- Up to 4 ml sample volume
- Total in-process control
- Advanced process safety and standardized sample preparation
- CE-marked according to IVD-directive*



**) In compliance with the Directive 98/79/EC on in vitro diagnostic medical devices (IVD-Directive). Products which are CE-marked according to the IVD-Directive can be used for diagnostic applications in countries where this directive is recognized. The device is not approved by the US FDA.*

FEATURES

- Heat lysis and heat elution
- LIMS connectivity
- Choice of elution tubes or plates
- Barcoded labware for complete sample traceability
- Plug-in for separate hand-held barcode scanner
- Drop catcher minimizes the risk of cross contamination
- UV light enables decontamination of the worktable

ORDERING INFORMATION

Product	Package size	Catalogue number
InviMag® Free Circulating DNA Kit/ IG	8 x 12 purifications	2439320400
InviMag® FFPE DNA Kit/ IG	8 x 12 purifications	2432120100
InviGenius® PLUS	1 piece	5011102000
InviQuant GeneCount 40	200 tests	3130100100

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molecular

STRATEC Molecular GmbH

Robert-Rössle Straße 10
13125 Berlin
Germany
Phone +49 30 94 89 29 01
Fax +49 30 94 89 29 09
molecular@stratec.com
www.molecular.stratec.com