Reference methods and materials for KRAS mutations in cell free DNA

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Overview

- Cell-free DNA (cfDNA) and liquid biopsy
- Candidate reference system for measurements of circulating tumour DNA (ctDNA)
- Development of plasmid reference materials for KRAS mutations
- Development of a digital PCR-based primary reference method for KRAS G12D copy number concentration
- Development of commutable calibrators for cfDNA analysis

Cell-free DNA

- Cell free DNA in blood circulation (plasma/serum)
  - Circulating DNA first identified in plasma 1948
- cfDNA present in other body fluids
  - Urine
  - Stool
  - Cerebrospinal fluid
  - Saliva
- Origin of majority of cfDNA: death of hematopoietic cells/ tissues; macrophage release

Mandel P, Métais P (1948) Les acides nucléiques du plasma sanguin chez l'homme. CR Acad Sci Paris 142: 241–243.

Challenges of cell-free DNA as a clinical analyte

- Typically low concentration
  - Typically 5-10 ng/mL plasma (~ 10^3 haploid genome equivalents (GE)/mL)
- Increased quantities of cfDNA observed in advanced cancers / during chemotherapy (up to 10^6 GE/mL)
- Circulating tumour DNA (ctDNA) is often the minority component
- Small genetic changes between normal/cancer:
  - Point mutations (single nucleotide variations (SNV))
  - Copy number variations

The Telegraph

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Blood test to replace repeat biopsies

A new "liquid biopsy" blood test could revolutionise cancer treatment by allowing doctors to track how tumours are responding to treatment without the need for surgery, a study claims.

BioSITrace

'Liquid biopsy' cancer diagnostics

- Screening
- Diagnosis
- Prognosis
- Monitoring treatment efficacy
- Drug resistance

➢ Forshew *et al* 2012. *Sci. Transl. Med*. Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA.

QIAGEN Announces First-ever Regulatory Registration of a Lung Cancer Companion Diagnostic Based on Liquid Biopsies

HILDEN, Germany, January 12, 2015 /PRNewswire/ --

- **QIAGEN's circulating tumor DNA test is now CE-IVD marked to assess EGFR mutation status in non-small cell lung cancer (NSCLC) patients based on plasma samples**
- ***therascreen EGFR RGQ Plasma PCR kit* helps physicians to identify patients who could benefit from treatment with IRESSA when tumor tissue sample is not evaluable**
- **QIAGEN pioneering the use of liquid biopsy-based companion diagnostics as a less-invasive option to complement surgical biopsies for genomic profiling of cancers**

QIAGEN (NASDAQ: QGEN; Frankfurt, Prime Standard: QIA) announced today the CE-IVD marking of its novel liquid biopsy-based companion diagnostic that analyzes circulating nucleic acids obtained from blood samples to assess an important genomic mutation in patients with non-small cell lung cancer (NSCLC), the most common form of this cancer.

The registration, which applies to more than 30 European countries, makes the new *therascreen* EGFR RGQ Plasma PCR kit the first-ever regulated companion diagnostic assay that has demonstrated clinical utility for guiding treatment decisions in patients with solid tumors based on the analysis of molecular biomarkers obtained from a body fluid (liquid biopsy).

The launch of this kit, which is planned for January 2015, comes as the European Medicines Agency (EMA) extended the drug label of IRESSA® to include the detection of EGFR mutations in circulating tumor DNA (ctDNA) obtained from a blood (plasma) sample when a tumor sample is not evaluable. This change was based on the IFUM (IRESSA Follow-Up Measure) study, which assessed the mutation status in tumor and circulating tumor DNA (ctDNA) samples derived from plasma using QIAGEN kits.

The *therascreen* EGFR RGQ Plasma PCR kit, co-developed by QIAGEN and AstraZeneca (LSE, NYSE and OMX: AZN), helps physicians to identify those advanced NSCLC patients who could benefit from treatment with IRESSA when a suitable tumor sample is

More by this Source

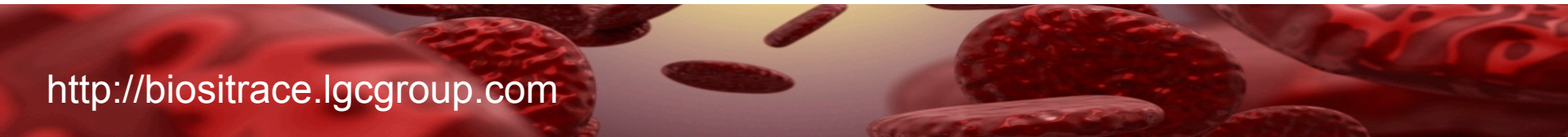
QIAGEN meldet Ergebnisse für das dritte Quartal und die ersten neun Monate 2015
Oct 28, 2015, 16:05 ET

QIAGEN Reports Results for Third Quarter and First Nine Months of 2015
Oct 28, 2015, 16:05 ET

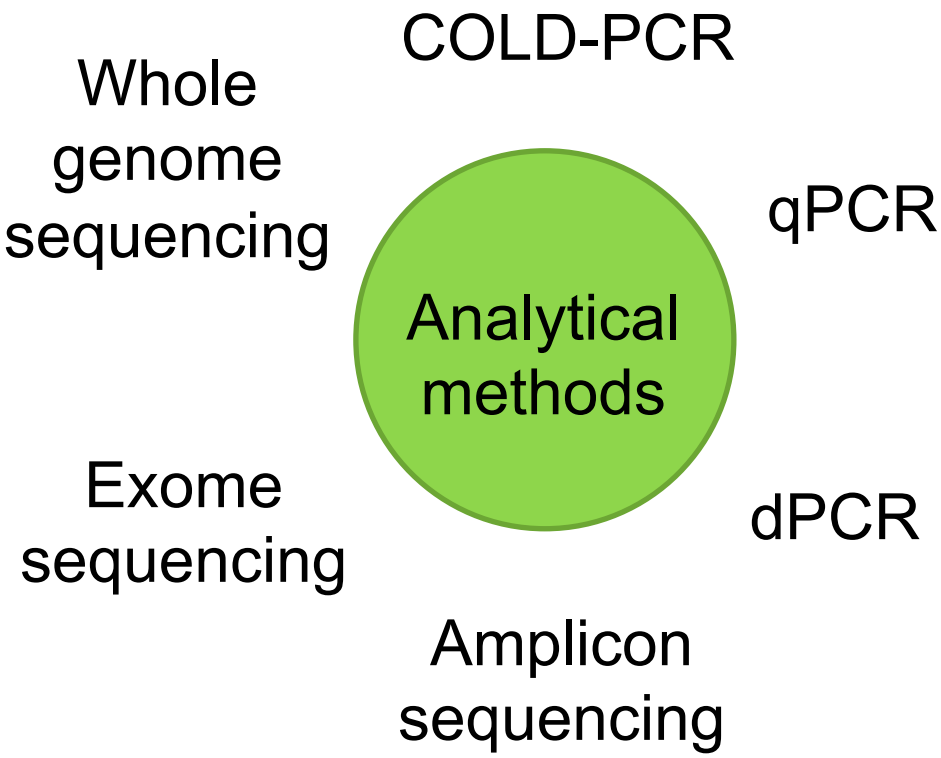
QIAGEN améliore l'accès mondial au dépistage avancé du cancer du col utérin
Sep 17, 2015, 09:00 ET

View all news by QIAGEN N.V.

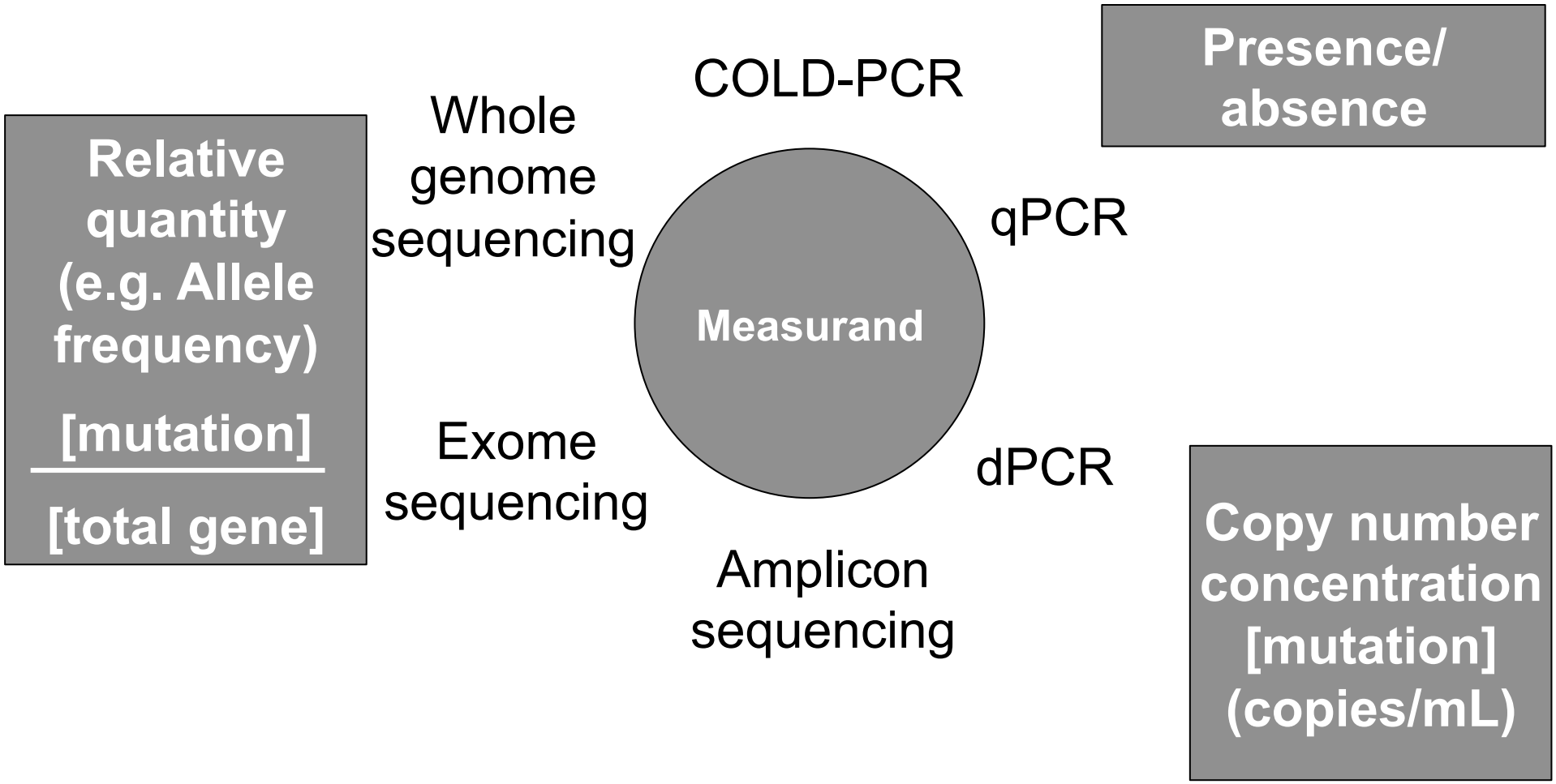
Journalists and Bloggers



ctDNA methods and measurands



ctDNA methods and measurands



Bio SITrace

Traceability for biologically relevant molecules and entities

Funded by the European Metrology Research Programme

Goal: Develop and verify methods for accurate counting for:

- Lipoproteins (LDL)
- Cells (CD4+ lymphocyte count, Circulating tumour cells)
- **DNA (Cell free DNA)**

Approaches to characterise the purity of biological reference materials

- NGS

LGC PTB TÜBİTAK INRiM ISTITUTO NAZIONALE DI RICERCA METROLOGICA UME NIB NATIONAL INSTITUTE OF BIOLOGY LNE Sharing a passion for progress

EURAMET European Association of National Metrology Institutes

group.com

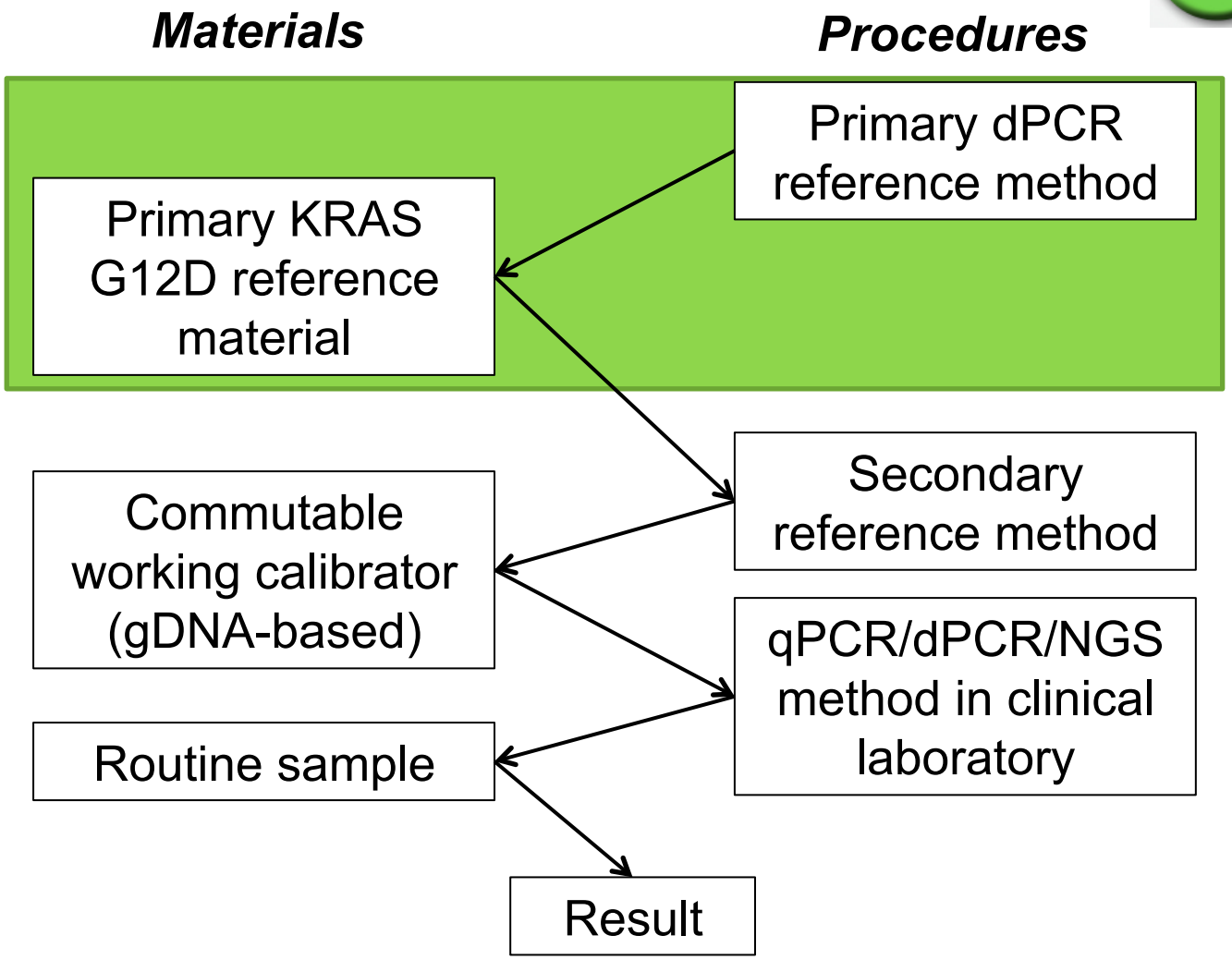
EMRP European Metrology Research Programme ▸ Programme of EURAMET

The EMRP is jointly funded by the EMRP participating countries within EURAMET and the European Union

Model: Mutant KRAS

- Mutant KRAS selected as model system for a clinically relevant biomarker
  - KRAS mutations are present in 40% of colorectal adenocarcinomas
    - G12D most frequently occurring mutation
  - predictive biomarker of non-response to anti-epidermal growth factor receptor (EGFR) antibodies
  - potential for non-invasive monitoring of tumour progression and drug resistance through circulating tumour DNA (ctDNA)
  - > 70 PubMed entries “KRAS ctDNA”

Measurand: KRAS G12D copy number concentration (Unit: copies/μL) (mol/l)



Primary KRAS reference materials

Key characteristics:

- Simple template: KRAS gene region targeted by molecular assay
- Highly purity (~100% KRAS single SNP)
  - ➢ Basis for defining assay interactions with potential interfering species such as other KRAS SNPs / pseudogenes within human genome
- Defined molecular weight
  - Suitable for analysis with orthogonal methods (for example, ID-MS, ICP-MS)
- Homogeneous with respect to template size
  - PCR amplification not influenced by DNA integrity

Primary KRAS G12D reference materials: KRAS constructs

```
G13D    AATATAAACTTGTGGTAGTTGGAGCTGGTGACGTAGGCAAGAGTGCCTTGACGATACAGC
G12V    AATATAAACTTGTGGTAGTTGGAGCTGTTGGCGTAGGCAAGAGTGCCTTGACGATACAGC
G12A    AATATAAACTTGTGGTAGTTGGAGCTGCTGGCGTAGGCAAGAGTGCCTTGACGATACAGC
G12D    AATATAAACTTGTGGTAGTTGGAGCTGATGGCGTAGGCAAGAGTGCCTTGACGATACAGC
WT      AATATAAACTTGTGGTAGTTGGAGCTGGTGGCGTAGGCAAGAGTGCCTTGACGATACAGC
G12R    AATATAAACTTGTGGTAGTTGGAGCTCGTGGCGTAGGCAAGAGTGCCTTGACGATACAGC
G12C    AATATAAACTTGTGGTAGTTGGAGCTTGTGGCGTAGGCAAGAGTGCCTTGACGATACAGC
G12S    AATATAAACTTGTGGTAGTTGGAGCTAGTGGCG  \G( CAAGAGTGCCTTGACGATACAGC
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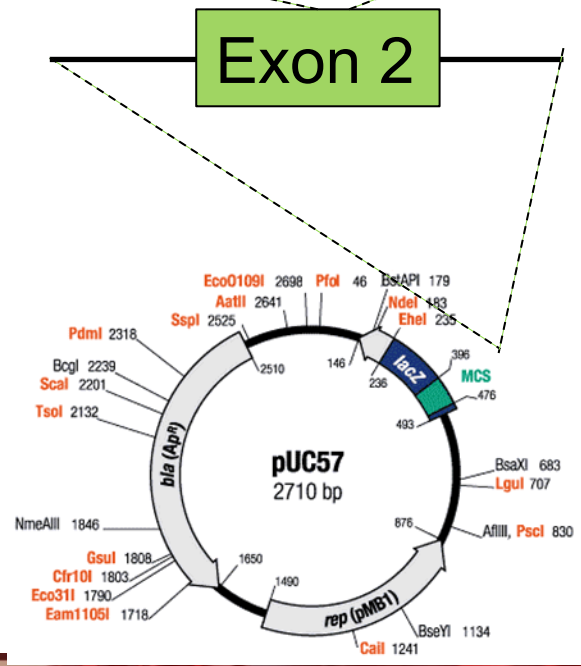
- Eight constructs:
  - gDNA sequence to include exon 2
    - 1624 bp consisting of intron 1, exon 2 (124bp) and intron 3
  - 7 pathogenic SNV mutations
  - 1 wild-type (WT)



dPCR as a primary reference method for KRAS mutations

Potential biases:

1. Assay chemistry
   - Are all template copies present amplified?
   - Molecular dropout (negative bias)
2. dPCR platform/technology
   - Are the concentration values from different instruments in agreement?
   - Bias/variation in partition volume

Measurement precision

- Inherent assay properties
- Platform properties (number of partitions)

Evaluation of assay chemistries

1. **Intercalating dye (EvaGreen)**
   - Detects total KRAS (both G12D and WT)
   - 80 bp and 164 bp amplicon sizes
2. **5' nuclease (Taqman) probe assays**
   - 2 probes specific to G12D and WT
   - 2 assays: PrimePCR (BioRad) and from literature (Taly et al, 2013)*
3. **Scorpion**
   - Primer-probes specific to G12D and WT

**Taly, V. et al. Clin. Chem. (2013). Multiplex picodroplet digital PCR to detect KRAS mutations in circulating DNA from the plasma of colorectal cancer patients.*

Development of commutable calibrators for cfDNA analysis

- KRAS plasmid DNA digested to *in vivo* cfDNA fragment size

Linearised plasmid (~ 4 kb)

186 bp fragment

- Materials prepared containing KRAS G12D and WT plasmid fragments
  - KRAS G12D minority fraction mimicking clinical scenario

Development of commutable calibrators for cfDNA analysis

- Plasmid fragment materials used as test materials in dPCR inter laboratory trial to evaluate:
  - Sensitivity
  - Specificity
  - Reproducibility
- Bio SI-trace NMI partners

- > 20 end-user diagnostic laboratories using Bio-Rad QX100™ /QX200™ droplet digital PCR system

Development of commutable calibrators for cfDNA analysis

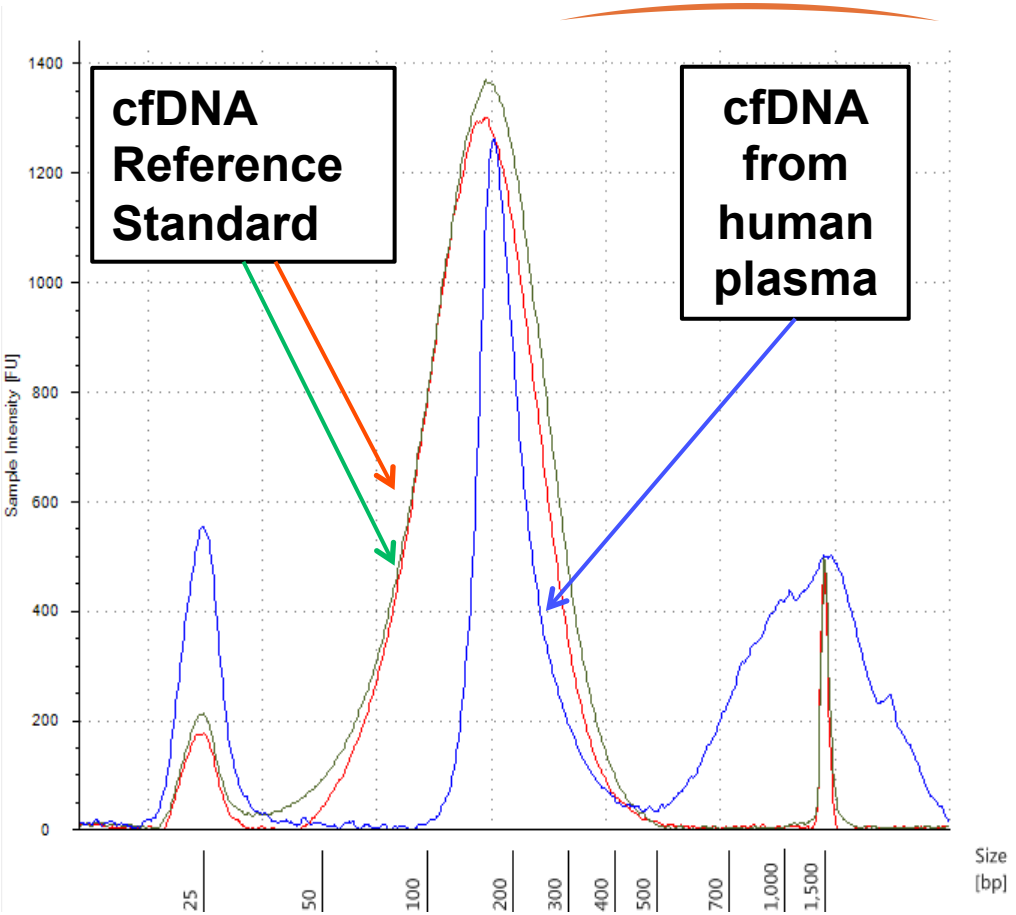
Project: Enabling stratified medicine through cell and tissue reference standards for minimally invasive cancer testing

Aims

1. Test the capability of dPCR to quantify tumour-associated mutations at low allelic frequencies in a complex gDNA template
2. Develop QC approaches for assessment of complete workflows for cfDNA analysis

Development of commutable calibrators for cfDNA analysis

- Horizon Discovery cfDNA Reference Standards
  - gDNA isolated from highly validated isogenic cell lines
  - fragmented by mechanical shearing (Covaris S220) to average size of 160 bp
  - Evaluated using Tapestation system (Agilent) (right)



Objectives

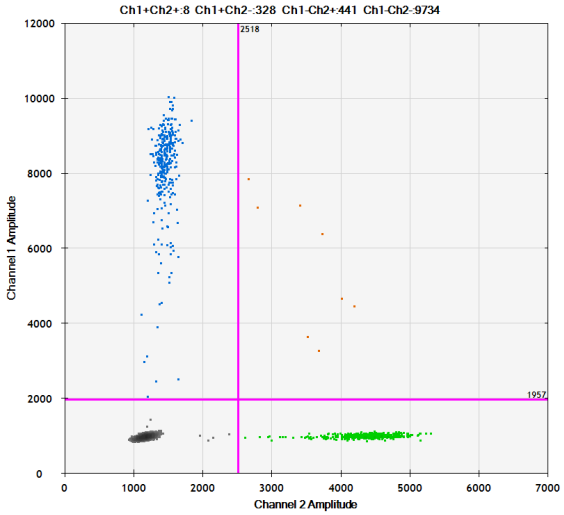
1. Analyse copy number concentration of cfDNA Reference Standard for mutant KRAS by dPCR
2. Assess utility of controls for calculation of efficiency of whole workflow for cfDNA analysis by spiking into plasma

cfDNA Reference Standard

Plasma

cfDNA extraction

dPCR analysis (QX200)



Properties of dPCR measurement and extraction process

Concentration (copies/μL (input))						Extraction efficiency
cfDNA Reference Standard (n = 9 reactions)			**Reference Standard spiked into plasma** (n = 6 extractions)			
Mean	**SD**	**%CV**	**Mean**	**SD**	**%CV**	**Mean**
2	1	59%	1	1	106%	40%
9	1	14%	4	2	36%	48%
38	6	16%	19	8	42%	50%
191	20	10%	103	33	32%	54%

Conclusions

- dPCR is a robust method for concentration measurements of mutant and wild-type KRAS DNA
  - EvaGreen, 5’ nuclease and Scorpion assay chemistries demonstrated comparable concentration estimates of total and MT KRAS independent of a calibration curve
  - Concentration estimates from 5 different dPCR platforms were within 20% of each other
  - New assay designs (primers/probes) and chemistries should be validated in a dPCR format
- dPCR methods can be applied to complex gDNA templates and demonstrate good within-laboratory reproducibility

Summary

- The Bio-SITrace project has systematically evaluated factors influencing the accuracy and precision of dPCR using the reference KRAS panel
- Results indicate that dPCR has the potential to be a highly reproducible reference method
  - Enabling calibration of reference materials
  - Supporting proficiency testing of diagnostic methods
  - Enabling comparison between datasets from different platforms and laboratories, assurance of analytical reproducibility and translation of these new methods to assist in patient care
  - Allowing regulatory / accreditation compliance for testing laboratories

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