HORIZON DISCOVERY

Diagnostic Reference Materials & Controls

Jonathan Frampton
Horizon Discovery

Comparability of Horizon ctDNA artificial plasma to real samples

Real Plasma samples

Unknown allelic frequency; copies/µl

Variable quantity and concentrations

Lot-to-lot variability

Irregular supply

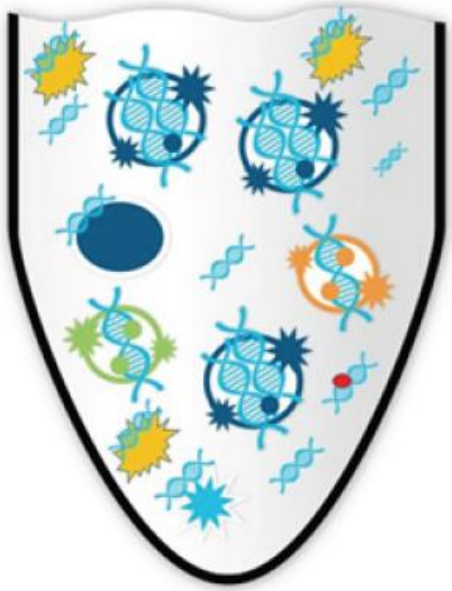
Limited supply of genotypes

Logistical challenges

Variable contamination with other analytes/genomic DNA

ctDNA degradation –time-limited storage

Difficult to determine extraction efficiency



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Precisely defined allelic frequency; copies/µl

Defined volume and concentrations

Lot-to-lot stability

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Availability of rare genotypes

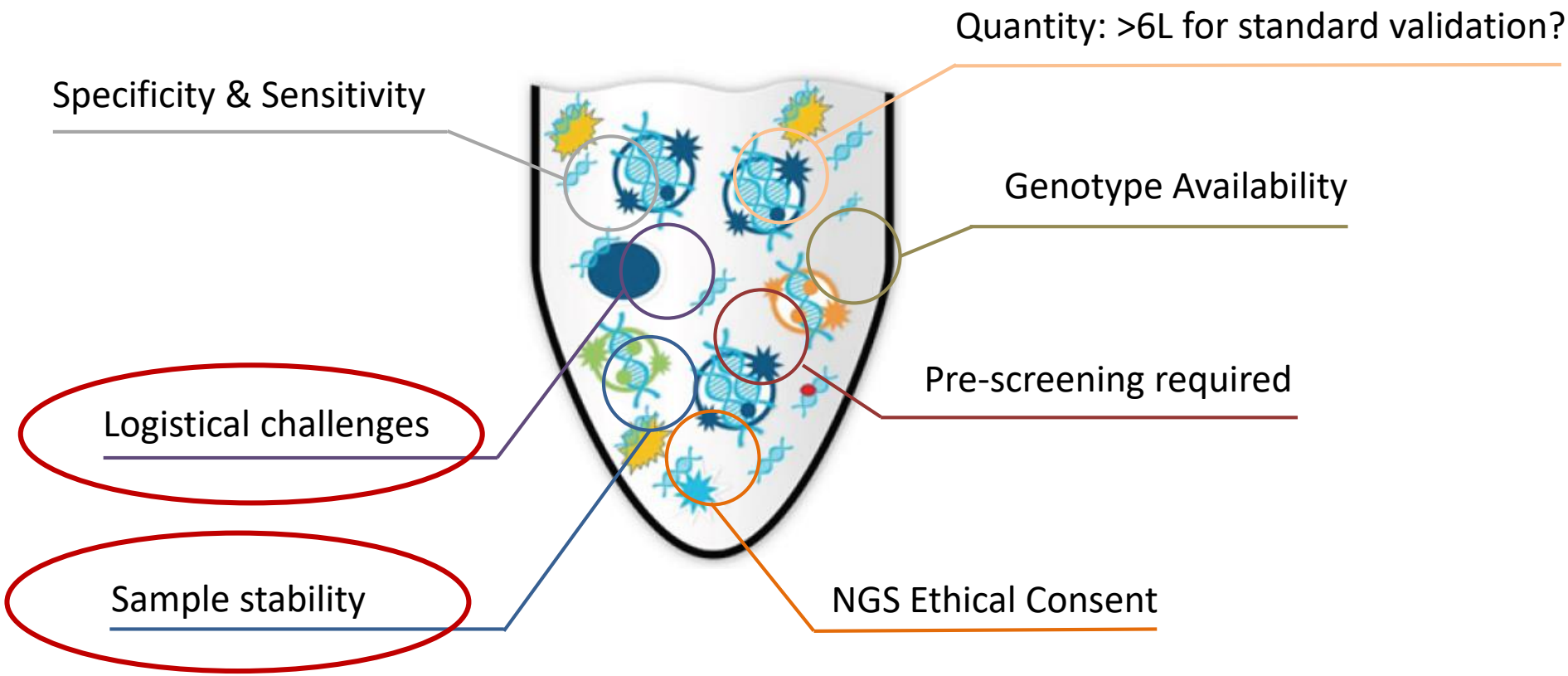
Standard shipping procedures

No interfering contaminants/analytes/genomictDNA

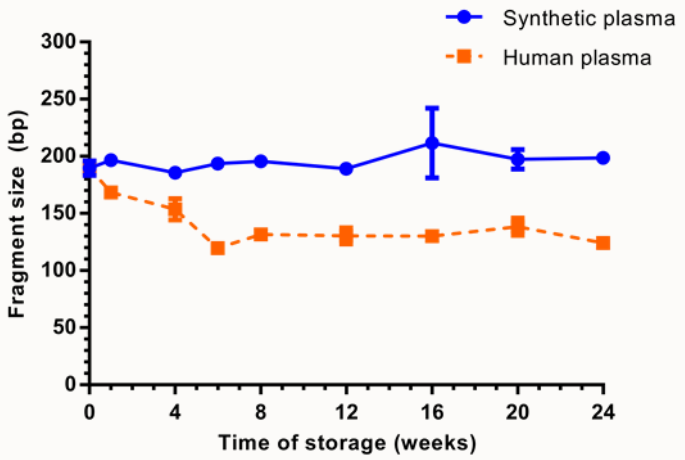
Long –term stability of ctDNA

Measurable and reproducible extraction efficiency

Horizon's artificial reference standards are ideal for analytical development validation & supporting global proficiency testing schemes.


Quantity: >6L for standard validation?
Specificity & Sensitivity
Genotype Availability
Pre-screening required
Logistical challenges
Sample stability
NGS Ethical Consent

Storage, Shipping & Stability

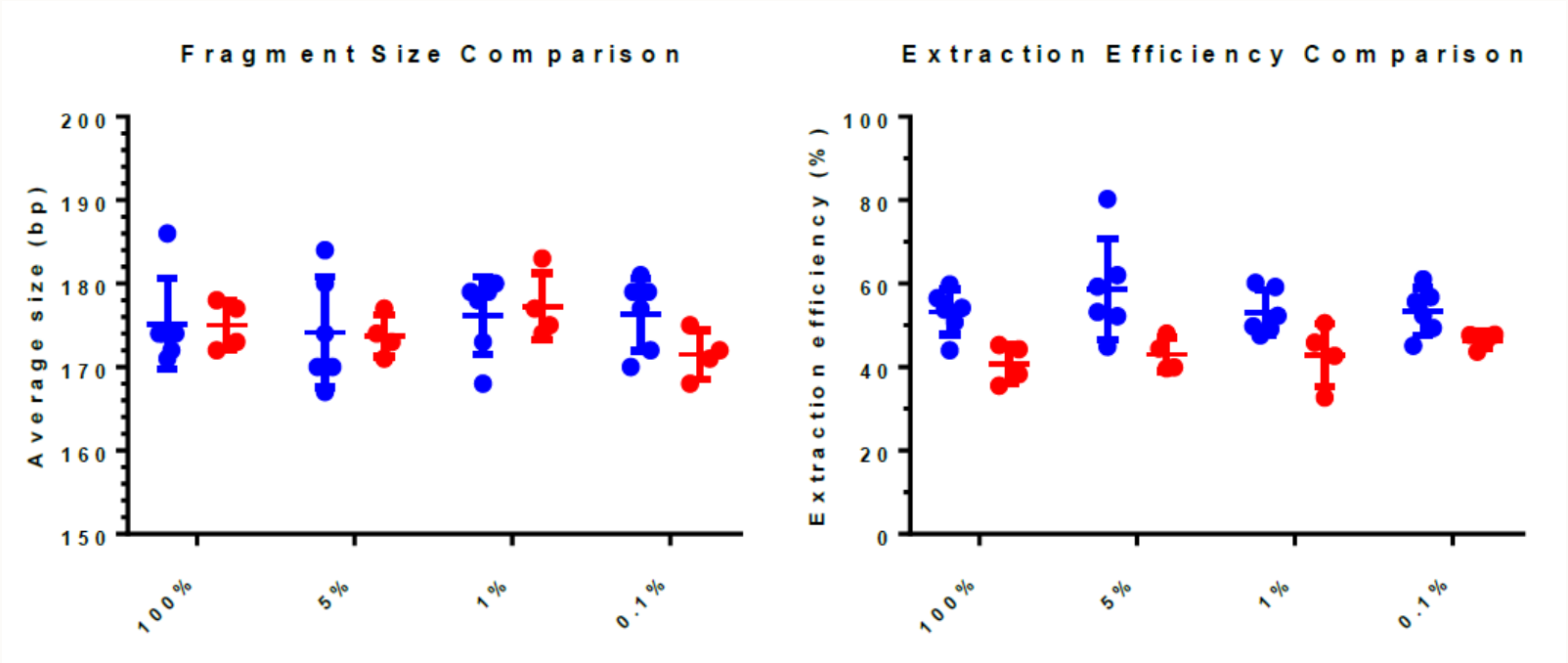


Storage at -80°C

ctDNA artificial plasma

- Stable at 4°C for 12 months from manufacture
- No pre-screening required
- Ease of logisitics worldwide (ex. HTA)
- Full NGS consent for analysis
- Large volumes easily available

Shipping on dry ice (-80°C) vs blue ice (4°C)



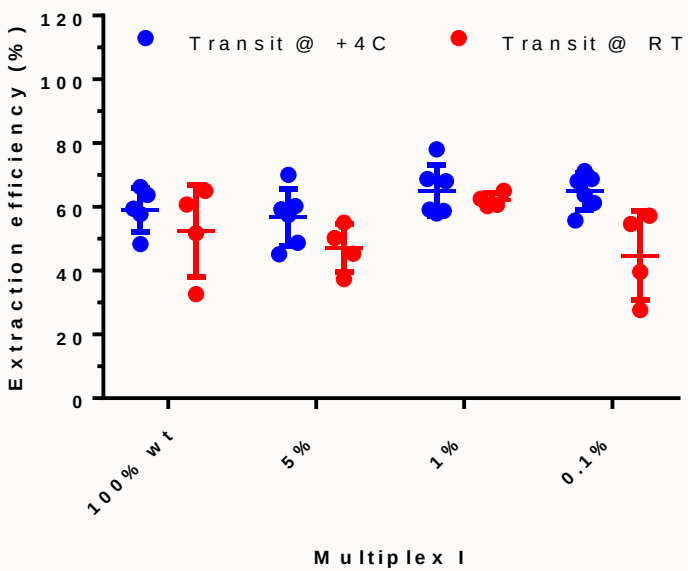
- Transit test 4°C for 5 days;
- Transit test -80°C for 5 days;

Shipping temperature does not impact DNA fragment size or stability
Artificial ctDNA reference standards are stable for 4°C shipments

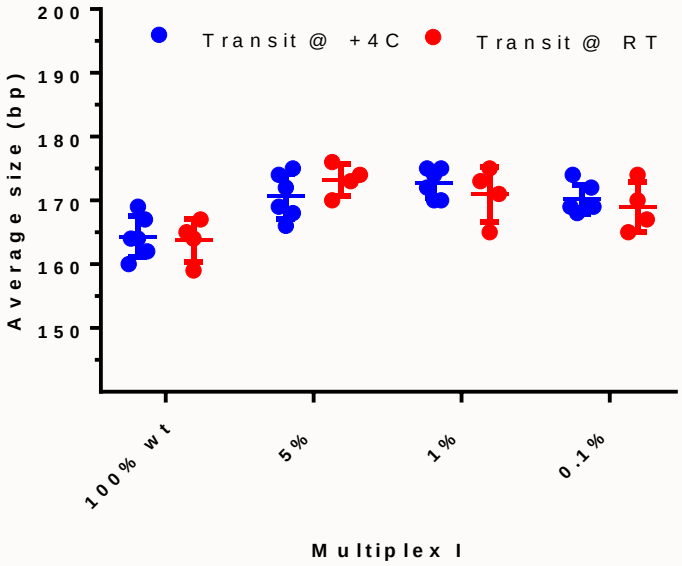
Shipping at RTP vs blue ice (4°C)

Transit of artificial plasma

Extraction efficiency



Fragment Size



ctDNA artificial plasma Horizon samples can be shipped at RTP

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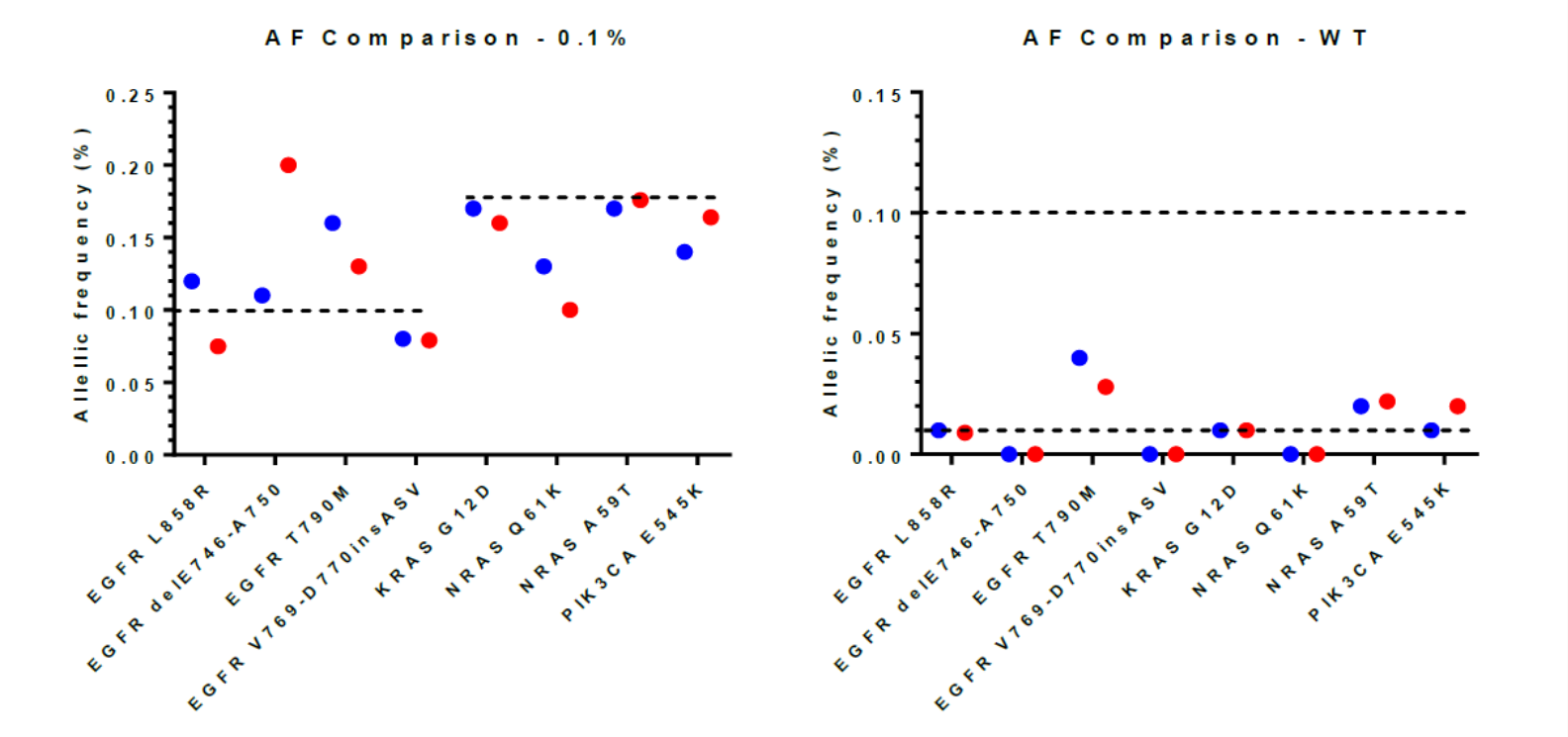

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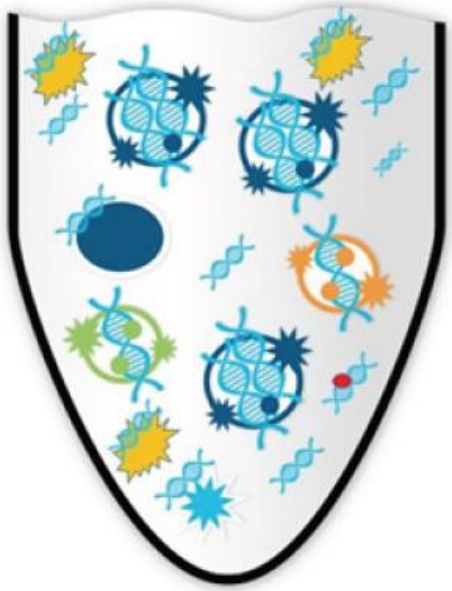

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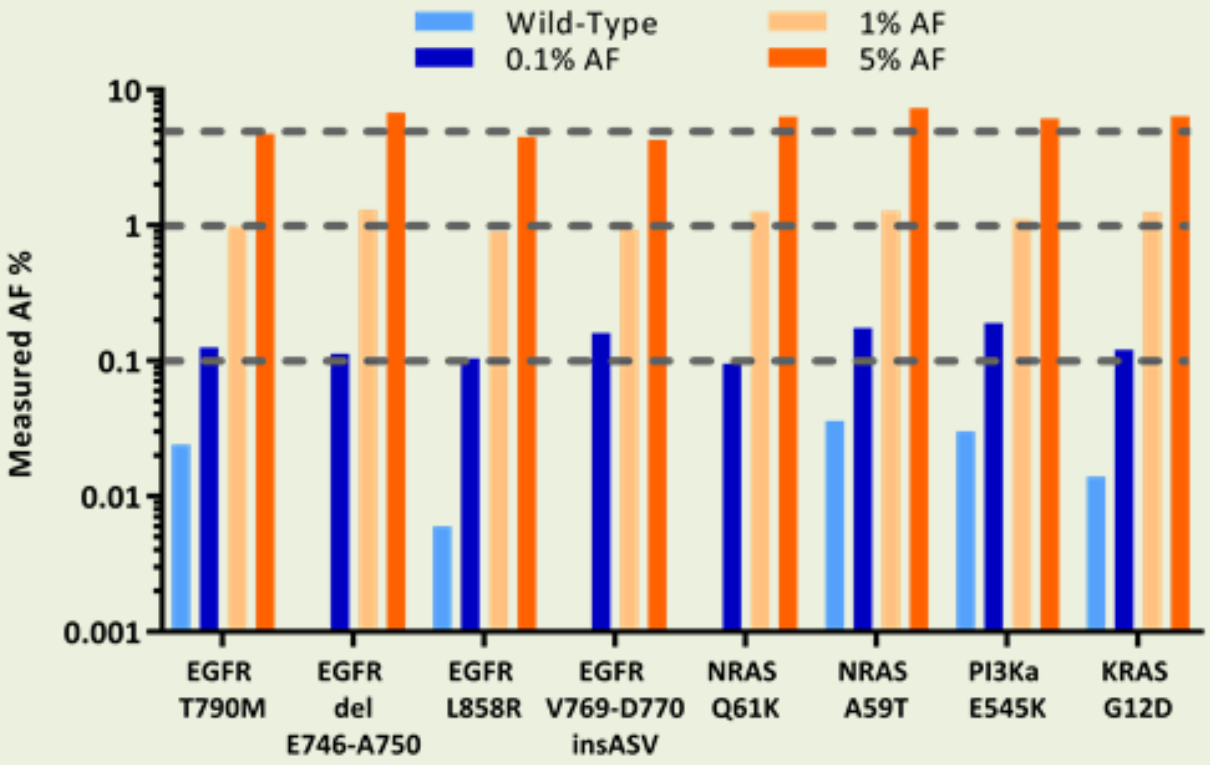
Critical Specifications & Comparability

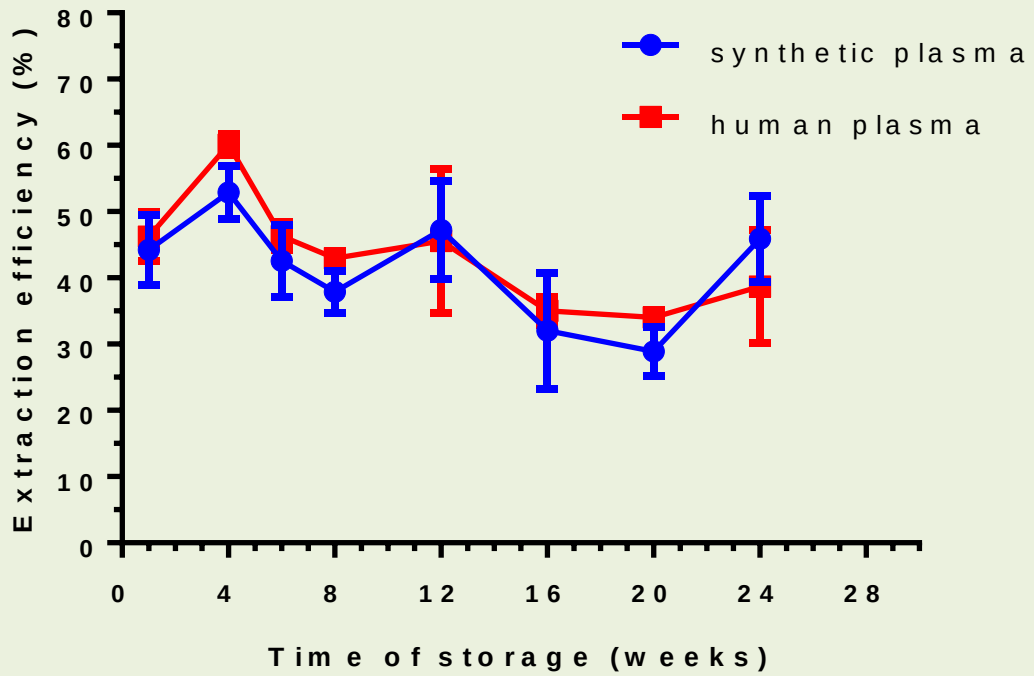
Horizon's ctDNA Allelic Frequency capabilities



Can provide ctDNA reference materials as low as 0.1% allelic frequency

Critical Specifications & Comparability

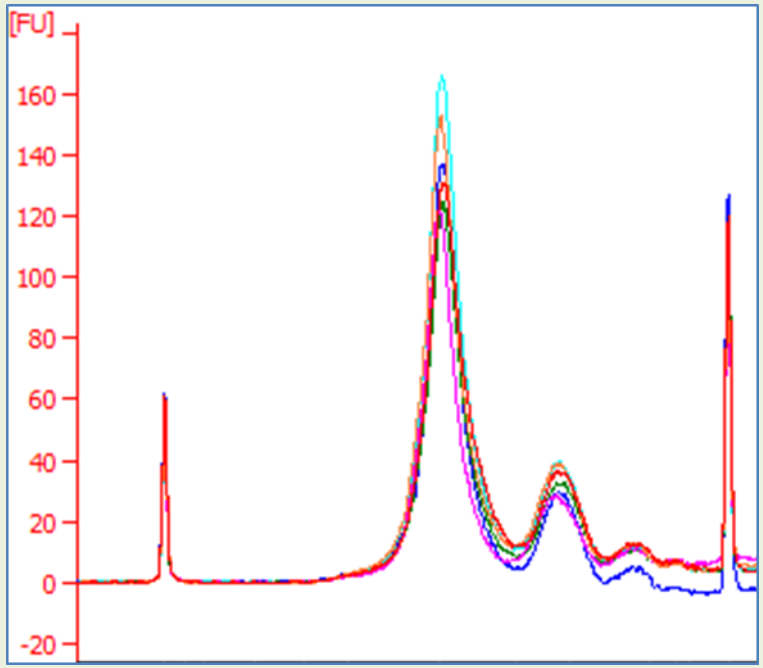
Comparability of ctDNA extraction



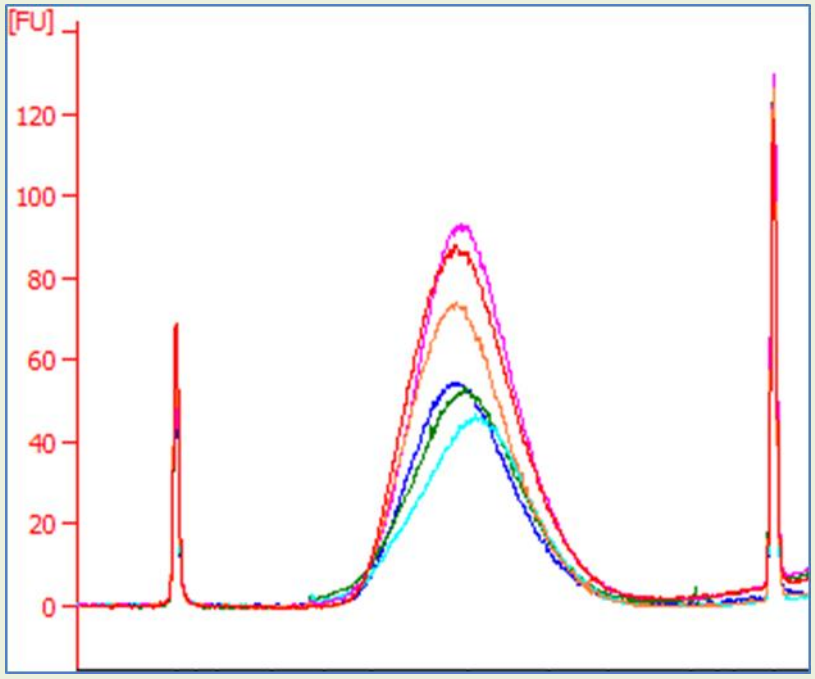
Comparable DNA extraction from plasma or artificial plasma

Critical Specifications & Comparability

ctDNA Fragment Size



Patient ctDNA

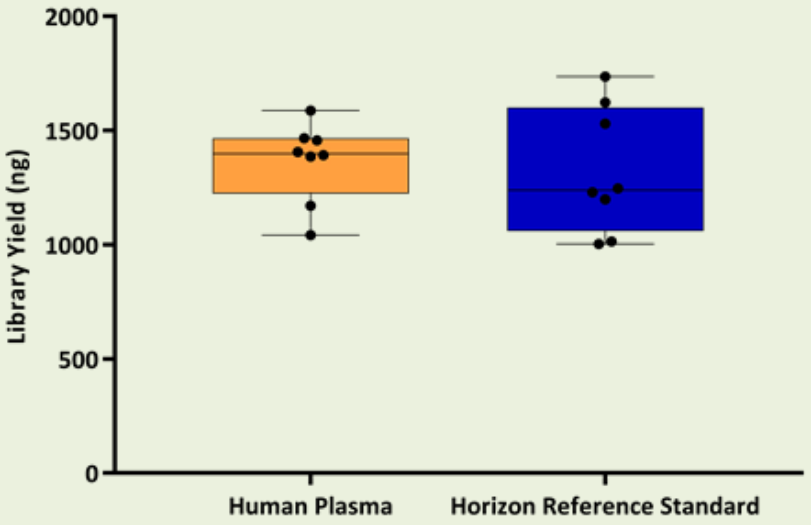


Horizon ctDNA

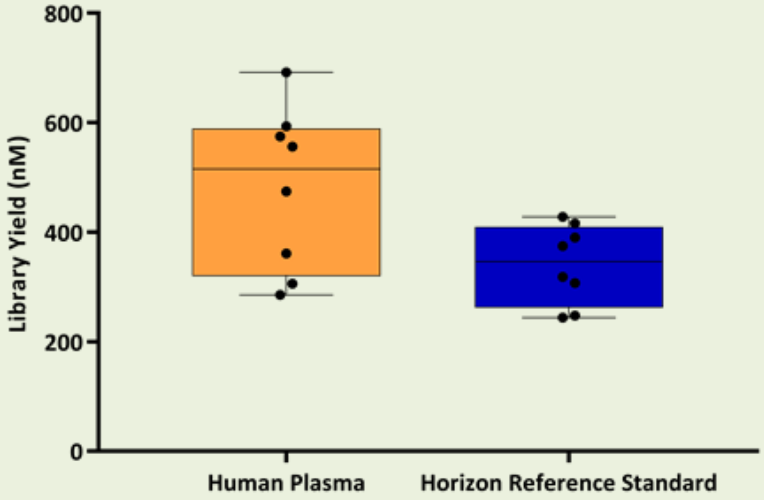
Comparable DNA fragmentation profiles between real plasma ctDNA & Horizon samples

Critical Specifications & Comparability

Comparability of ctDNA extraction



qPCR quantification



Picogreen fluorescence

Comparable library preparation between real plasma ctDNA & Horizon samples

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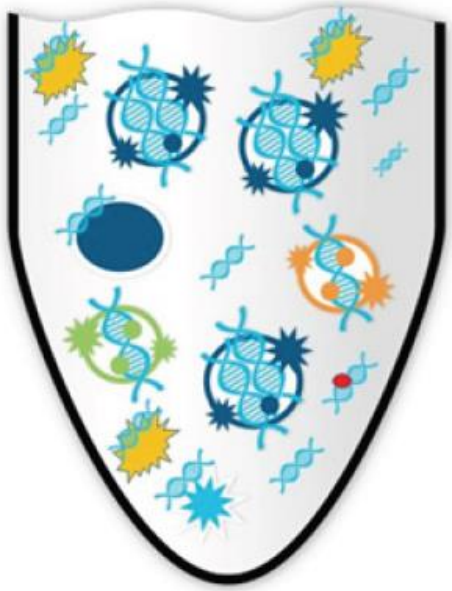

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What does the cfDNA Multiplex Plasma Reference Standard look like?

		Expected Allelic Frequency (AF%)			
Gene	**Variant**	**5% Multiplex I**	**1% Multiplex I**	**0.1% Multiplex I**	**100% WT Multiplex I**
EGFR	L858R	5.00	1.00	0.10	0.00
EGFR	ΔE746 - A750	5.00	1.00	0.10	0.00
EGFR	T790M	5.00	1.00	0.10	0.00
EGFR	V769 - D770insASV	5.90	1.00	0.10	0.00
KRAS	G12D	6.30	1.30	0.13	0.00
NRAS	Q61K	6.30	1.30	0.13	0.00
NRAS	A59T	6.30	1.30	0.13	0.00
PIK3CA	E545K	6.30	1.30	0.13	0.00

Scope and design of the study

Evaluation of the performance of circulating tumor DNA (ctDNA) sequencing provider panels

ctDNA Samples

12 blinded 2 ml ctDNA reference plasma samples

- 6 replicates
- pre-defined allelic frequencies of validated genomic alterations

NGS Providers

4 ctDNA NGS providers (selected out of 11)

using multi-gene panels consisting of 50 - 70 genes

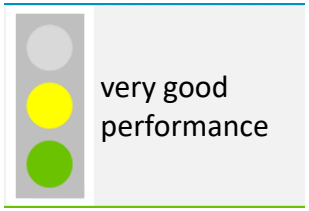
Data Analysis

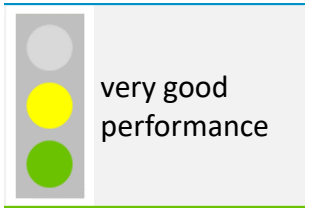
Comparison of detected genomic alterations

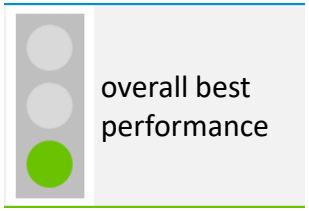
Guidance for the implementation of ctDNA sequencing into clinical trials

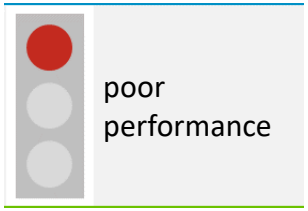
Summary

Provider	A	B	C	D
DNA extraction efficacy (mean)	45 %	60 %	73 %	43 %
Replicate performance & precision	3rd	2nd	1st	poor
Accuracy 100 validated mutations	85 %	79 %	95 %	-
Detection of 434 confirmed mutations	77 %	86 %	93 %	-
Number of CNA	33	10	59	-
Frameshift mutations	0	38	109	-
Rearrangements	8/8	4/4	8/8	-









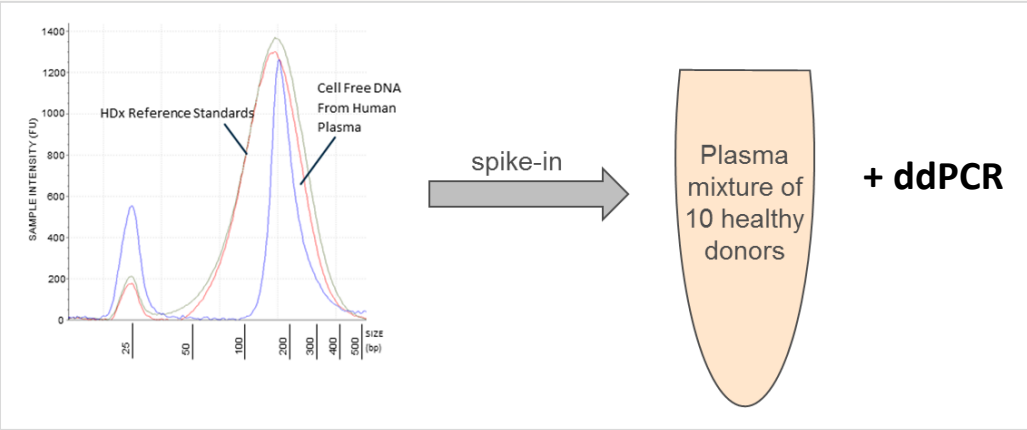
ctDNA reference standards are suitable as internal proficiency controls

Publication in progress; evaluation performance of providers by using ctDNA reference & patient samples

Generation of ctDNA reference samples at Horizon Discovery

Horizon Discovery reference standards spiked into human plasma from consenting donors

- fragmented human genomic DNA (average size 170 bp) derived from engineered human cell lines
- mechanical shearing method



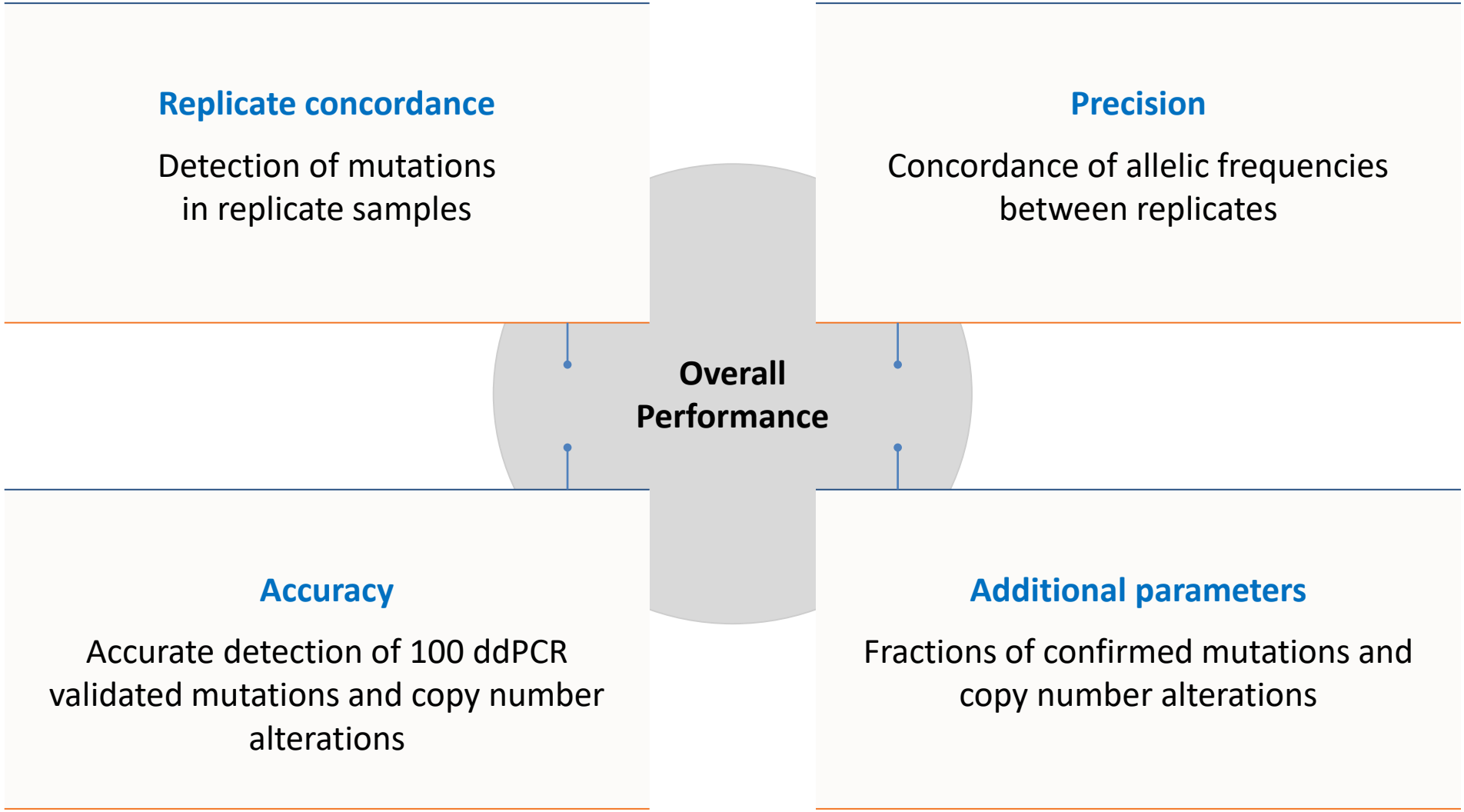
I) 4 replicates of Multiplex I cfDNA reference standard

- Digital droplet PCR (ddPCR) validated mutations of 4 genes: EGFR, KRAS, NRAS, PIK3CA
- Allelic frequencies 5%, 1%, 0.1% and matched wild type

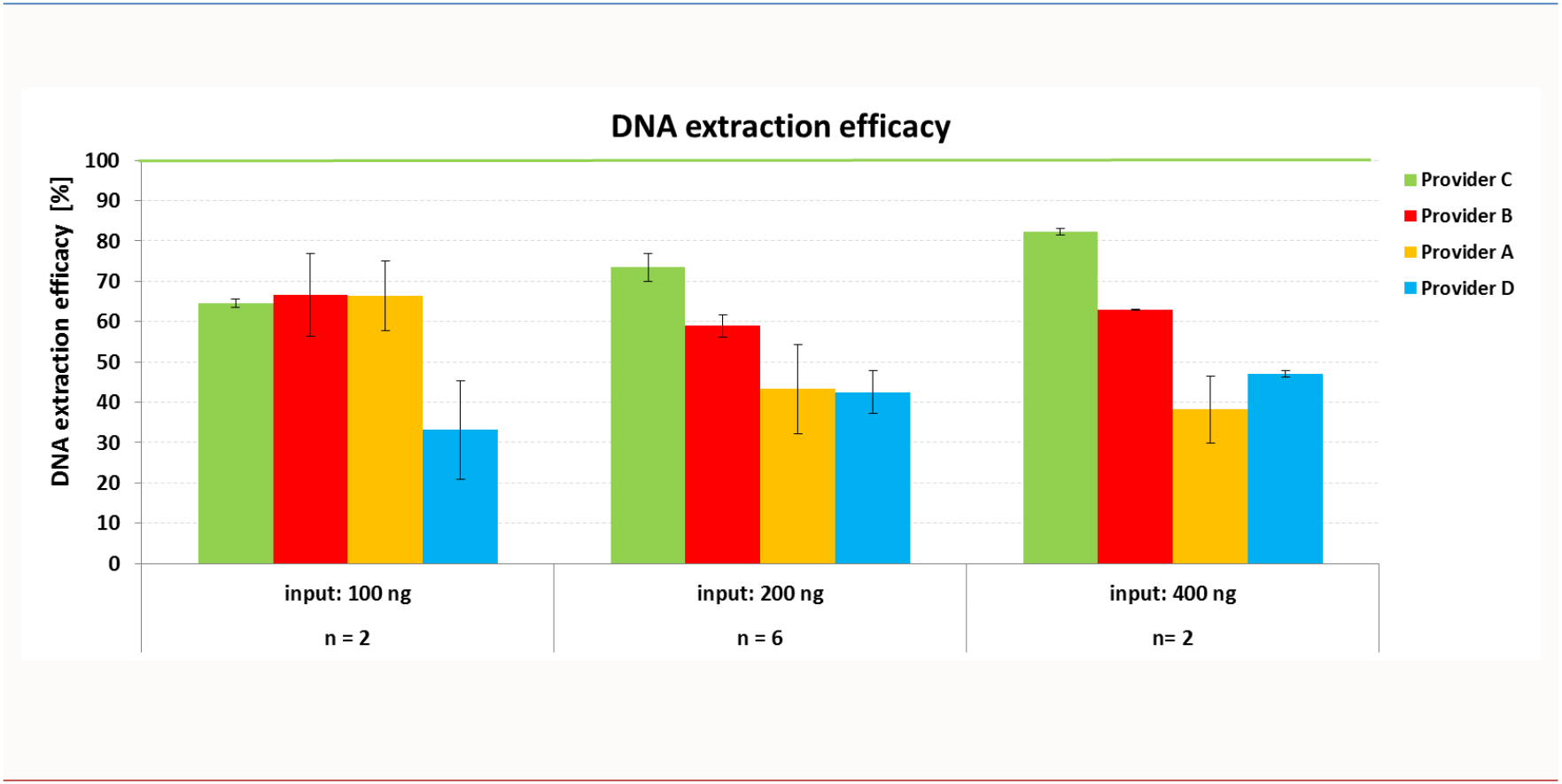
II) 2 replicates of Structural Multiplex cfDNA reference standard

- ddPCR validated mutations of 8 genes: AKT1, EGFR, GNA11, MET, MYC, PIK3CA, RET, ROS1
- Range of allelic frequencies
- Short nucleotide variants (SNVs), copy number amplifications (CNA) and rearrangements

Criteria to determine the overall performance



DNA Extraction



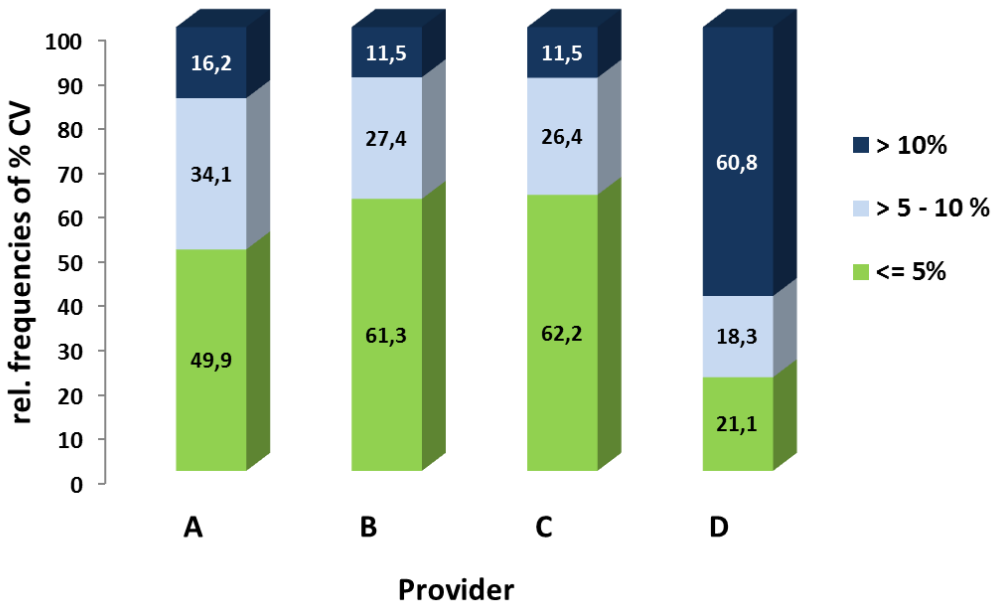
Different extraction methods impact total DNA yield
Increased DNA input impact extraction efficicacy

SNVs & SNPs

Precision

- Providers A, B and C reported the majority of replicates (> 85 %) with good precision (% CV < 10 %)
- Results of Provider D showed a high variability (% CV > 10%) for ~ 60% of the values

Relative frequencies of % CV levels



Overall high precision by Providers A, B and C

SNVs & SNPs Nomenclature

Genomic sequence (hg19, GRChr37):

```
7:55248998 ATGGCCAGCGTGGACAAC 552490015
             M  A  S  V  D  N
```

color change=possible ins site

Result after insertion: a short duplication

```
              8        9        10
insertion: ATGGCCAGCGTGGCCAGCGTGGACAAC
insertion: ATGGCCAGCGTGGCCAGCGTGGACAAC
            M  A  S  V  A  S  V  D  N
           766767                770
```

AA change:	766M > MASV	
position:	55248999 (9)	
AA change:	p.Ala767_Val769dup	
position:	**55248998 (8)**	
AA change:	D770_N771>ASVDN	
position:	**55249010 (10)**	

Identical mutation reported in 3 different ways
→ Comparison requires harmonization of results

Performance of detecting 100 validated mutations and CNAs

Accuracy

- no false positives (16 of 16 wt)
- false negatives only at low mutation allele frequencies and copy number amplifications

	False negative rate	Number of false negatives				
		SNVs 0.1 %	SNVs 1%	SNVs 5%	Met CNA at CN ~ 3	MYCN CNA at CN ~ 7
Provider A	16 %	13	0	0	1	0
Provider B	21 %	14	4	0	2	0
Provider C	6 %	5	0	0	0	na

Provider C reported most accurate results

Detection of frameshift mutations

Frameshift (fs) mutations

- Provider C: 109 >> Provider B: 38 >> Provider A: 0

# fs	Provider B	Provider C	
17	2/2	2/2	Provider B and C concordantly reported 17 fs mutations in both replicates at similar allelic frequency
4	1/2	2/2	Provider C detected 4 fs mutations in both replicates (dup), whereas Provider B reported those in only one of the replicates
29	0/2	2/2	Provider C consistently detected another 29 fs in both replicates at similar AF that are present in parental cell lines
9	0/2	1/2	

Provider C performed best:
Consistent replicate concordance and confirmation by a 2nd Provider

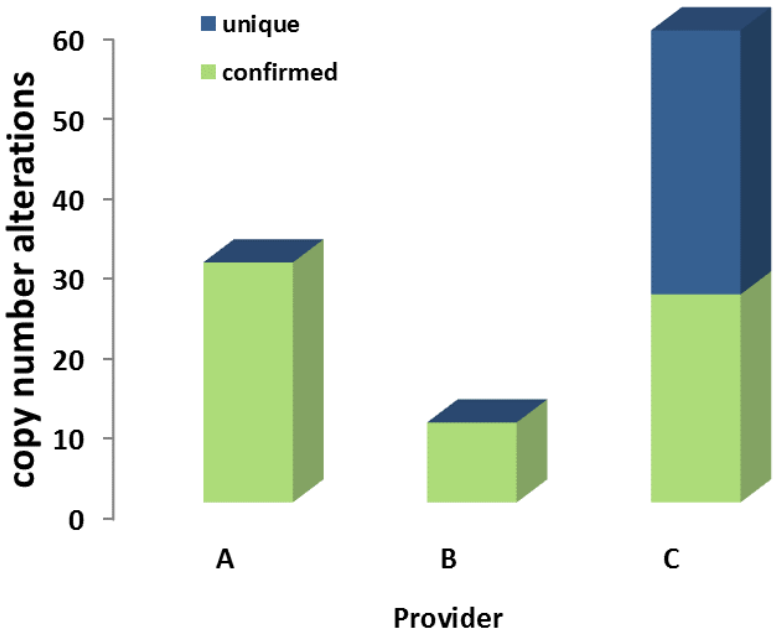
Detection of copy number alterations

Copy number alterations (CNAs)

- a total of 66 unique CN alterations were reported in 9 genes:

  Provider A → 33
  Provider B → 10
  Provider C → 59

- Provider C: consistent replicate performance for all 59 reported CNAs
- Provider A & Provider B:
  82 & 80% concordance of replicates



Provider C performed best: consistent replicate performance and highest accuracy (validated CNAs or confirmation by a 2nd Provider)

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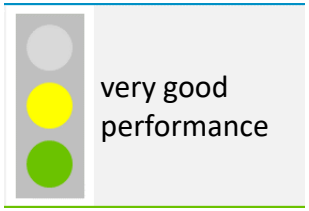

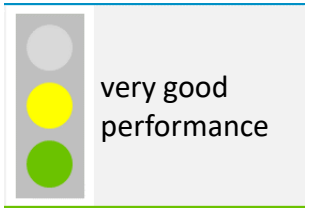

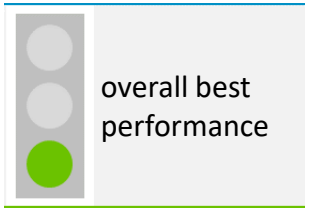

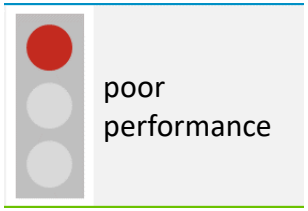

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