Frequently Ask Questions:

cfDNA Pan-Cancer Reference Standards v2

Background cfDNA, ctDNA

What is your background cfDNA made from?

The Twist wild-type background cfDNA mimic is cell-line-derived and treated with our proprietary method for closely mimicking the fragmentation profile of naturally occurring cfDNA, which is characterized by a sharp mononucleosomal peak and a secondary dinucleosomal peak

Could there be some mutation 'contaminants' coming from the background DNA as well?

The Twist background cfDNA high-fidelity non-amplified DNA is derived from our proprietary fragmentation process, and we have observed an extremely low mutation rate compared to competitor material.

Is your background cfDNA limited supply?

The Twist cfDNA is sourced from an immortalized cell line, which enables us to create large production lots with consistent lot to lot genotypes and performance.

How do you perform quality control on the reference standards?

Twist runs target enrichment NGS on the 0% and 5% VAF to ensure non-detection and detection, respectively, at all variant sites. Additionally, six sites across all VAF levels are confirmed via ddPCR.

Are these control oligos, RNA, what is in it/ components?

The Twist cfDNA controls are double stranded DNA. They are a mixture of synthetic "ctDNA" molecules designed and printed with Twist technology and spiked into the background cfDNA to form each specific VAF level.

How do you design the ctDNA/ variant list?

Twist performed a literature search to arrive at a list of curated, clinically-relevant (*i.e.* actionable) cancer-associated variants, as well as using the COSMIC database as a reference, where matching entries are available It includes the many prevalent variants in adult solid tumors. The 456 variants occur in 85 unique genes. Twist also separately annotates the set of clinical relevant variants (>140 of variants).

The configuration of variants and their precise reference and alternative sequence are listed on the Twist website.

What is the molecular structure of the 3′ and 5′ termini?

The variant fragments are blunt-ended, double-strand DNA molecules.

Twist has tested the material to be a suitable standard in non-fragmenting end-repair and A-tail ligation (ERAT) library preparation protocols, as well as in amplicon protocols, such as ddPCR and qPCR. The performance of the v2 Pan-cancer Reference Standard is similar to the v1, and to any other dsDNA input into library prep.

Were the mutations in the standards confirmed by NGS as well, as with digital PCR as it says in the document?

All 456 variants are confirmed by NGS (for both 0% and 5% VAF), and the 0.1%, 0.25%, 0.5%, 1% and 2% are generated from a blending of 0% and 5% stocks. We selected a set of six representative and relevant variants across 0% to 5% VAFs for ddPCR, showing quantitative formulations and a continuous serial dilution.

Experiment instruction, QC (NGS, ddPCR)

Recommendations for diluting the controls?

Twist recommends using "Low-EDTA TE," which is 10 mM Tris pH 8, 0.1 mM EDTA. If EDTA must be avoided for compatibility with downstream steps, Qiagen Elution Buffer (10 mM Tris-HCl, pH 8) can be used.

Can’t find indel or other hard to detect variants?

The standard contains many difficult-to-detect variants, including structural variants and INDELS ranging from 1 bp to 30 bp. Detection of individual variants can depend on what aligner and alignment setting the customer is using. These hard to detect variants are for experienced customers using their pipeline. So just having an understanding of it. SNVs and small INDELs (*e.g.* 5 bp or smaller) are more straightforward for customers to detect.

What reagents (*e.g*, library prep) do you use for the liquid biopsy workflow?

Cell-free DNA is generated in a highly specific biological process that yields a characteristic fragment length profile. Thus, we recommend preparing a library that does not change the size profile in any way: using the "Twist Mechanical Fragmentation Library Prep Kit" without any additional fragmentation treatment. Our library insert sizes look superb when we use that method.

We have used Twist UMI adapters extensively on the cfDNA samples, and they work great for duplex consensus and getting good assessments of the mutation rates.

What do you recommend for starting input?

Twist recommends starting with 30 ng of starting input. 20-30 ng is a typical range for liquid biopsy assay.

Do you recommend pooling this control with a patient sample after library prep, before NGS?

Once prepared into a library with indices, there are no issues with pooling this control with patient samples for simultaneous sequencing.

The variant (COSM214499) is always high in 0% in NGS QC results, why?

This deletion occurs in the gene ATM at a genomic context with 10 consecutive Ts. These consecutive Ts makes the locus a "slippery" sequence, where the DNA polymerase replicating a copy of the genome can have the template strand slip relative to the strand being synthesized, leading to an INDEL.

The same effect has a high likelihood of occuring by the same mechanism when a PCR polymerase copies the sequence during library prep PCR. Thus, this locus is especially likely to spontaneously form the variant sequence due to PCR cycling during ordinary library prep.

When the allele is not present at all, any human genomic sample (including the Pan-cancer Reference Standard) will show an elevated VAF for this mutation due to PCR error. (In pan-cancer 0% VAF, it shows up at a higher VAF than 99.8% of other variant sites in the standard.) Thus, the background noise is higher due to the genomic context, meaning the Limit of Blank (LoB) of this variant is likely higher than other loci due to the sequence contest.

When the allele is present, the true allele frequency will be added “on top of” the noise level of the locus, meaning this variant will likely have a higher Limit of Detection (LoD) than other variant sites.

Using UMI adapters (like the Twist UMI Adapters) and running consensus collapse and/or duplex collapse can help decrease the noise at this locus due to PCR error in library prep.

For NGS QC, do you use target capture sequence for the 456 variants, or use a whole genome sequence?

Twist performs whole exome sequencing for the background cfDNA background and utilizes a target capture on the 5% material, in addition to a ddPCR on the intermediate VAFs.

Would we still get a similar % AF at the range of 1 ng, 10 ng, 20 ng, 30 ng of input? And do we have any data if going lower how much it would impact the synthetic variants' AF?

The %VAF of the controls is a true statement about the bulk composition of the material. However, when taking a sample, especially a small sample, sampling "error", or variability, can be introduced to the experimental reading. A reduced input into an assay would reduce the abundance of both the mutant and wild-type molecules in the reaction.

Reduced sample input decreases the number of molecules potentially able to be incorporated into the library and detected by sequencing. When the number of mutant molecules gets low enough (approximately below 16 molecules), VAF metrics become subjected to Poisson sampling error, and so will show replicated-to-replicate variability in reported VAF (Hao, Y. et al., 2016).

The table below will provide an estimation of the copy number of variant DNA molecules in terms of input mass and genome equivalent copies for common low VAF levels.

ng DNA Input	Total Genomic Copies	2% VAF variant copies	1% VAF variant copies	0.5% VAF variant copies	0.25% VAF variant copies	0.1% VAF variant copies
1	300	6	3	1.5	0.75	0.3
10	3000	60	30	15	7.5	3
20	6000	120	60	30	15	6
30	9000	180	90	45	22.5	9

Product Configuration

456 variants are all in one tube, or subsets?

All 456 variants are stored in one tube.

What are the kit configurations of the cfDNA standards?

The full kit contains 7. If an individual tube is 3 ug, it is a single tube. The 2 tubes 300 ng configuration has 2 tubes.

Part Number	Description
107576	Twist cfDNA Pan-Cancer Reference Standard v2 Set, 300 ng kit
107577	Twist cfDNA Pan-Cancer Reference Standard v2 VAF 0% (WT), 3 ug
107578	Twist cfDNA Pan-Cancer Reference Standard v2 VAF 0.1%, 3 ug
107579	Twist cfDNA Pan-Cancer Reference Standard v2 VAF 0.25%, 3 ug
107580	Twist cfDNA Pan-Cancer Reference Standard v2 VAF 0.5%, 3 ug
107581	Twist cfDNA Pan-Cancer Reference Standard v2 VAF 1%, 3 ug
107582	Twist cfDNA Pan-Cancer Reference Standard v2 VAF 2%, 3 ug
107583	Twist cfDNA Pan-Cancer Reference Standard v2 VAF 5%, 3 ug
107584	Twist cfDNA Pan-Cancer Reference Standard v2 VAF 0%, 600 ng
107585	Twist cfDNA Pan-Cancer Reference Standard v2 VAF 0.1%, 600 ng
107586	Twist cfDNA Pan-Cancer Reference Standard v2 VAF 0.25%,600 ng
107587	Twist cfDNA Pan-Cancer Reference Standard v2 VAF 0.5%, 600 ng
107588	Twist cfDNA Pan-Cancer Reference Standard v2 VAF 1%, 600 ng
107589	Twist cfDNA Pan-Cancer Reference Standard v2 VAF 2%, 600 ng
107590	Twist cfDNA Pan-Cancer Reference Standard v2 VAF 5%, 600 ng