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# STUDY OF MRD

## *FLT3* ITD MRD Testing

Minimal residual disease (MRD) detection in patients with leukemia has proven to be useful in the clinical management of disease and can facilitate the development of new therapies. Mutations in fms related tyrosine kinase 3 (*FLT3*) gene are the most common mutations found in acute myeloid leukemia (AML) (NEJM. 2013; 368(22): 2059-74) and are characterized by an aggressive phenotype with a high prevalence of relapse. Internal tandem duplication (ITD) mutations within the juxtamembrane domain are the most common mutations in the *FLT3* gene (Expert Opin. Ther. Targets. 2015; 19(1): 37-54). The development of a sensitive and specific assay for *FLT3* ITD mutations represents a significant advancement in guiding treatment decisions.

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Invivoscribe's *FLT3* ITD MRD test is an NGS-based assay that was developed with accompanying bioinformatics software under full ISO 13485 design control, supporting regulatory clearance throughout the world. The assay was validated using cGMP quality testing controls to detect ITDs ranging from 3 bp to 126 bp in size; however, clinical performance of the assay has confirmed ITDs ranging from 3 bp to over 200 bp in size can in fact be detected. While we prefer to test a primary sample to identify the specific ITD (length and sequence) to be tracked in subsequent samples, our assay can detect ITDs - even if a diagnostic sample is not available. This assay can track *FLT3* ITDs at a sensitivity of $10^{-4}$ or lower, provided sufficient DNA quantity with desirable quality is available.

## 1| *FLT3* ITD MRD Clinical Testing Service

The *FLT3* ITD MRD Test developed by Invivoscribe is an amplicon-based next-generation sequencing assay. Provided that sufficient DNA is available for testing, this assay can detect mutations with a mutant cell sensitivity of $10^{-4}$ (1 mutant cell in a background of ten thousand normal cells; equivalent to an allelic sensitivity of $5x10^{-5}$ when a single mutant allele is present). Recommended sample types include peripheral blood, bone marrow, or high-quality extracted and purified genomic DNA (quantified with a method specific for double-stranded DNA). It is especially important that DNA be free of PCR amplification inhibitors when a high quantity of DNA is required (> 500 ng) for detection of *FLT3* mutations at low frequencies.

The following three controls are included in every test:

1. A positive control with an allelic concentration of $5x10^{-5}$ (approximates a mutant cell concentration of $10^{-4}$)
2. A negative control with a wild-type *FLT3* gene
3. A no template control (NTC) with water in place of the DNA sample in the PCR reaction

The sequencing output data is analyzed using an Invivoscribe developed proprietary *FLT3* ITD MRD Data Analysis Tool (MRDDAT).

Currently, *FLT3* ITD MRD testing is provided as a Research Use Only (RUO) service through Invivoscribe; however, the assay will soon be validated as a CLIA-approved assay and testing will be performed by the Laboratory of Personalized Molecular Medicine® (LabPMM®), a subsidiary owned and operated by Invivoscribe in San Diego, CA; Martinsried, Germany; and Tokyo, Japan.

## 2| DNA Input Quantity

The amount of input DNA interrogated and the total number of output sequencing reads are two critical parameters to consider for MRD testing. Routine tracking of a mutant at a sensitivity of $10^{-4}$ can be confidently achieved by detecting 1 mutant cell in a background of ten thousand cells, by testing as little as 500 ng of input DNA **(Figure 1A)**.

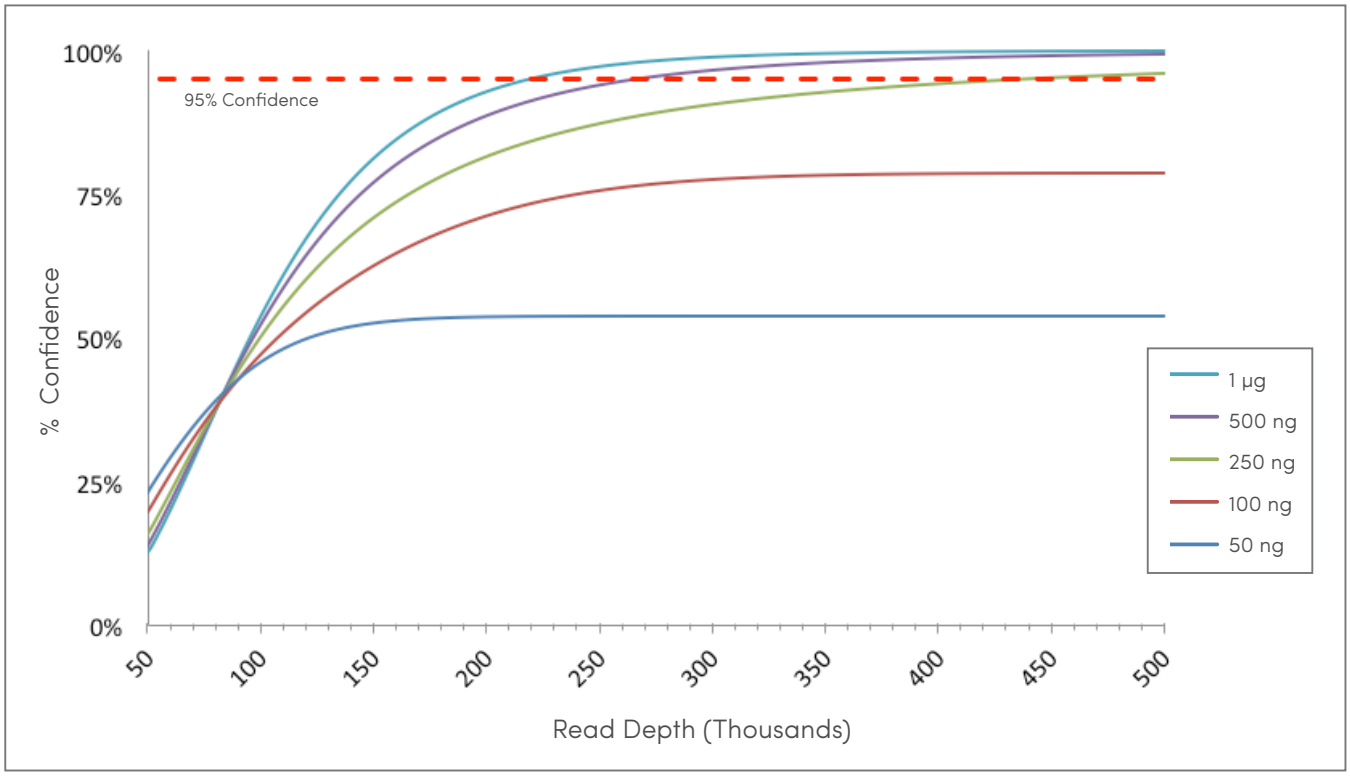
If sensitivities higher than $10^{-4}$ are desired, it is important to note that there are approximately 6.5 pg of DNA in each cell. Therefore, to detect 1 mutant cell in the background of a million normal cells at a confidence level of 95% (detection at $10^{-6}$) it is necessary to test approximately 35 µg of genomic DNA. Such a high level of sensitivity, although seemingly attractive, may not be required in most settings, and is beyond the level that is generally accepted to be the threshold indicative of residual disease ($10^{-4}$ - $10^{-5}$). In **Section 3**, relationships between DNA input, sequencing read depth, and confidence levels for different detection limits are illustrated.

## 3| Relationship Between DNA Input, Read Depth & Confidence Levels

The following figures **(Figure 1A, 1B and 1C)** depict the level of confidence for detecting a mutant (detected with at least 5 reads) at various DNA input quantities and replicates as a function of the number of sequencing reads obtained. The threshold (red-dotted line) is set at a 95% confidence level.

Note: The confidence levels for detecting *FLT3* mutations depicted in Figures A-C were calculated using a statistical model. This model does not incorporate PCR bias and, consequently, the calculated confidence levels are theoretical and not empirically determined.

**FIGURE 1A.** CONFIDENCE LEVELS FOR DETECTING *FLT3* ITD MUTATIONS AT $10^{-4}$, TESTING VARIOUS DNA QUANTITIES AS A FUNCTION OF READ DEPTH



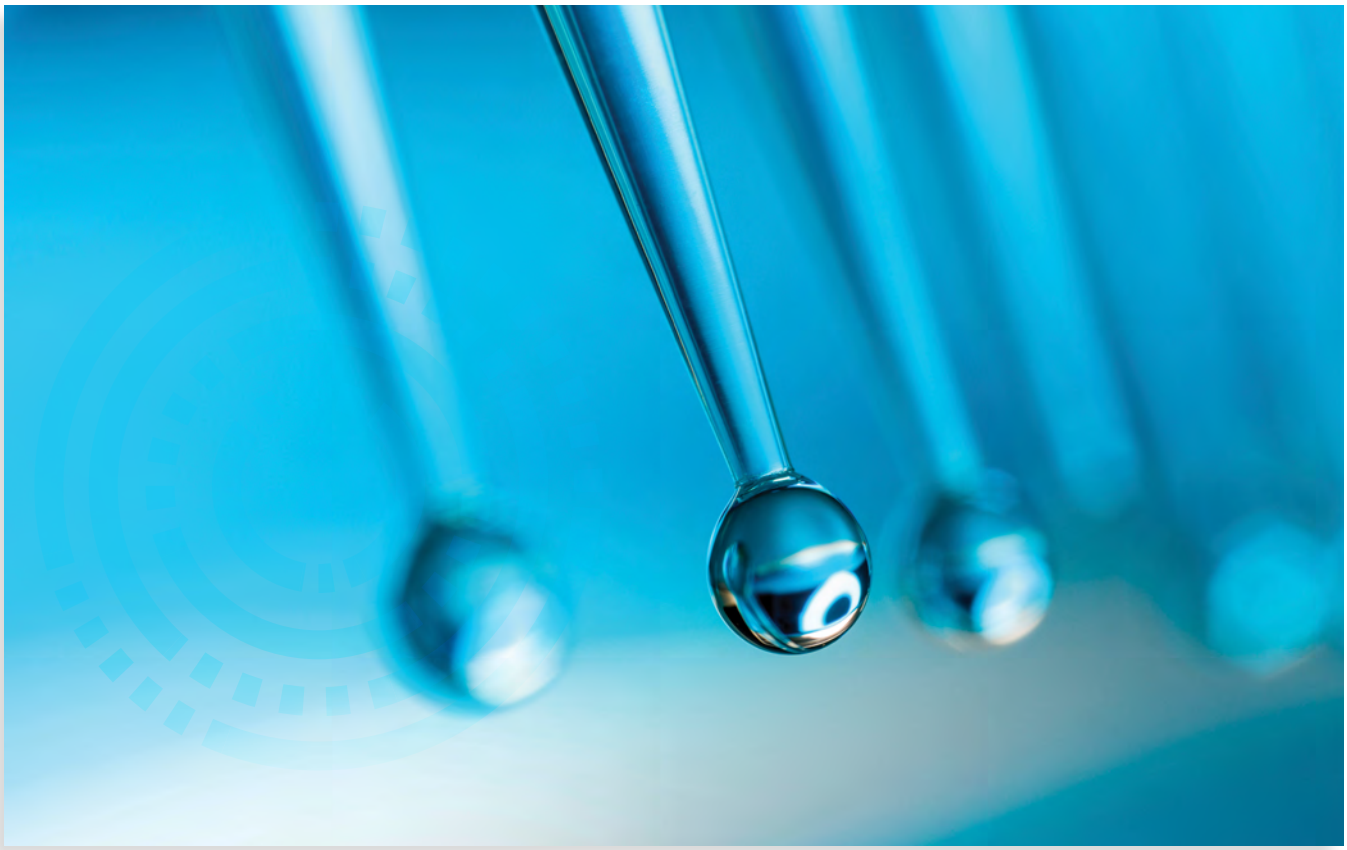



**FIGURE 1B.** CONFIDENCE LEVELS FOR DETECTING *FLT3* ITD MUTATIONS AT $10^{-5}$, TESTING VARIOUS DNA QUANTITIES AS A FUNCTION OF READ DEPTH

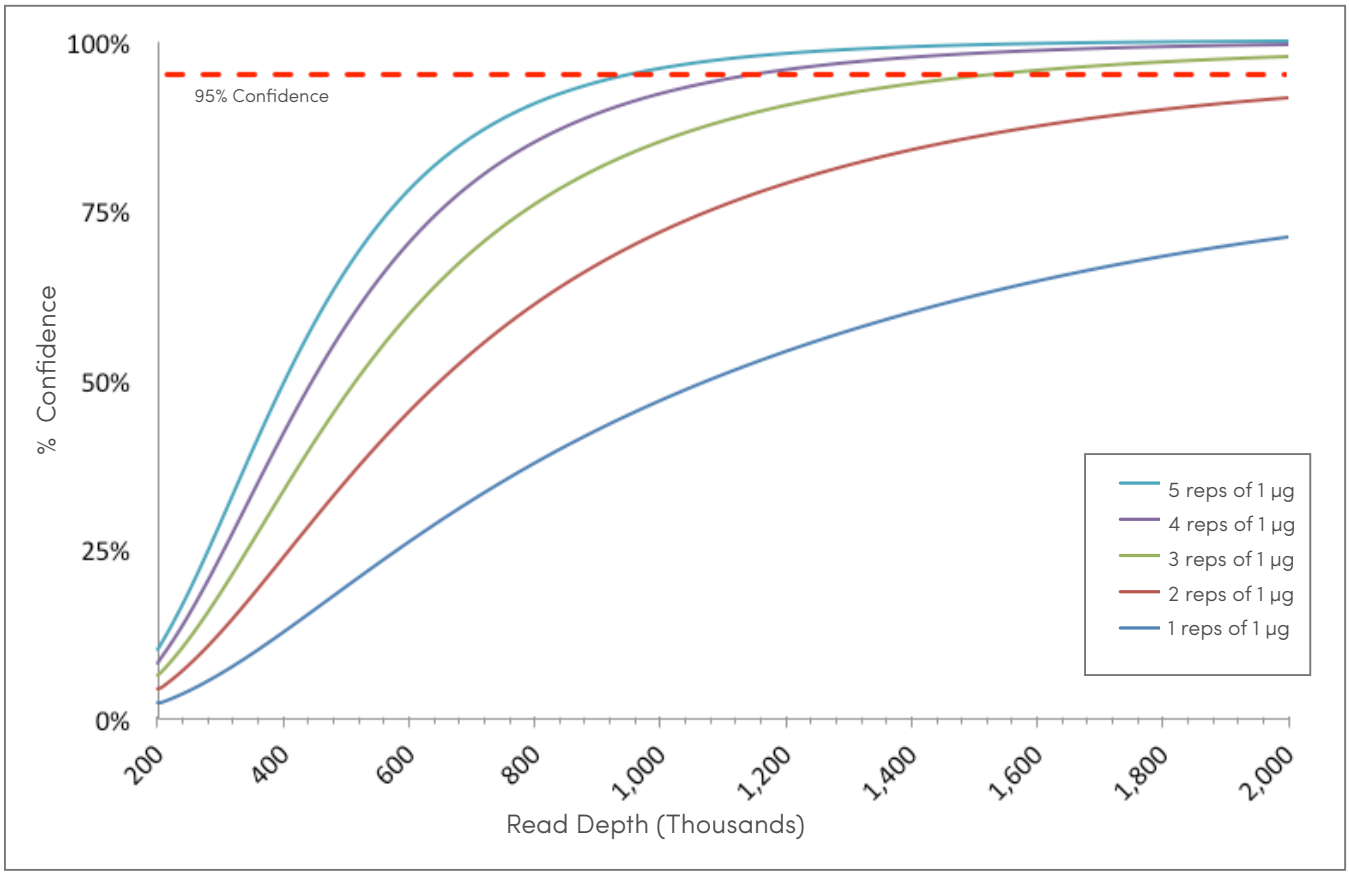


**FIGURE 1C.** CONFIDENCE LEVELS FOR DETECTING *FLT3* ITD MUTATIONS AT $10^{-6}$, TESTING VARIOUS DNA QUANTITIES AS A FUNCTION OF READ DEPTH

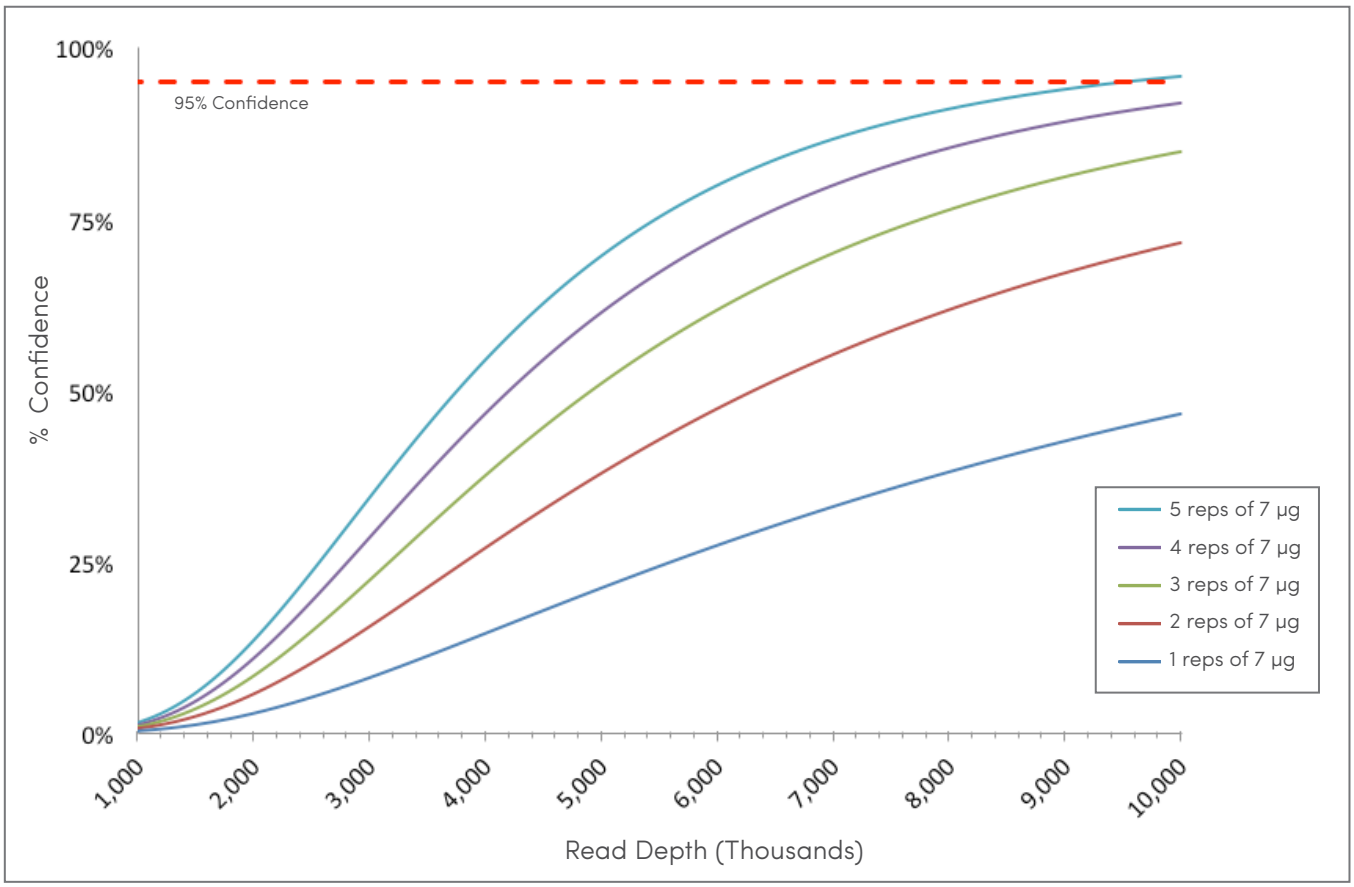
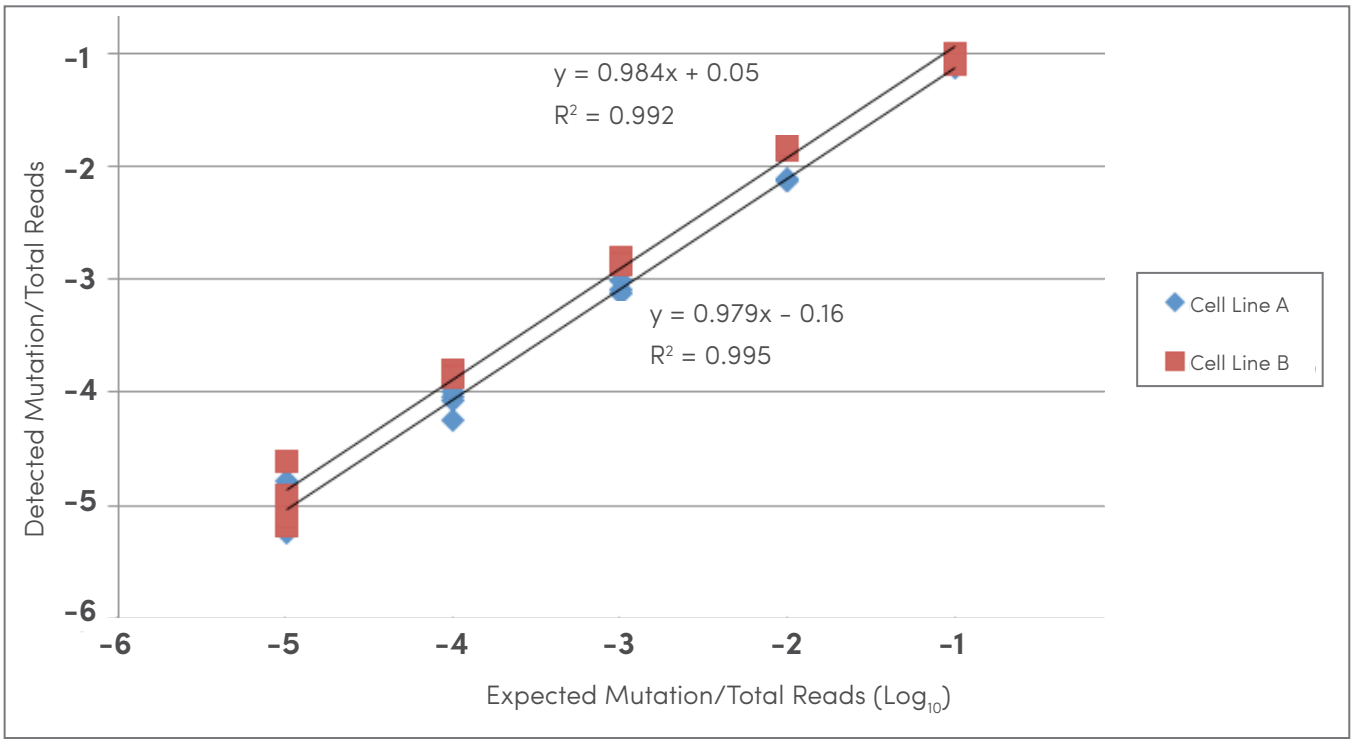
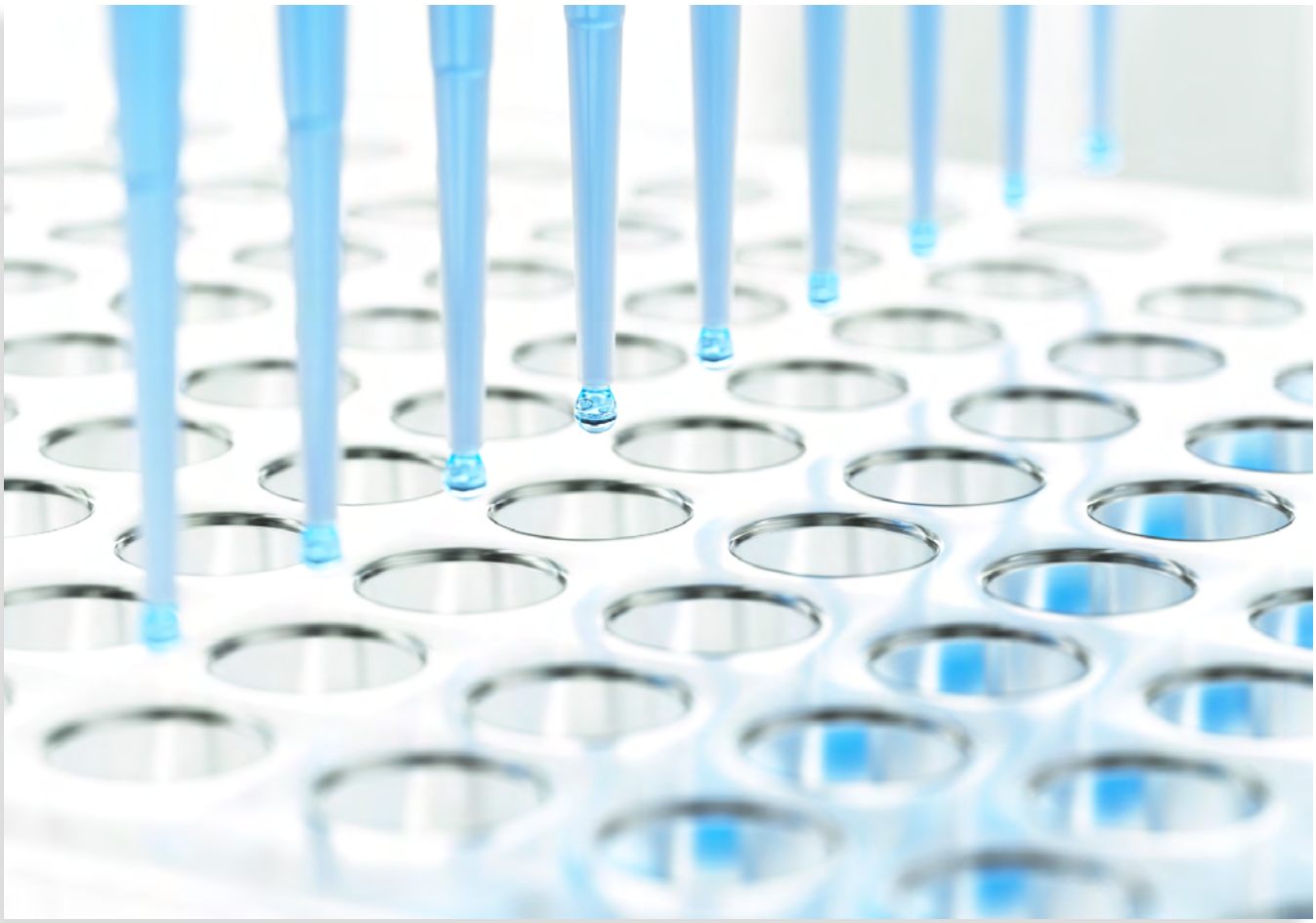



**FIGURE 2:** LINEARITY OF THE *FLT3* ITD MRD ASSAY



## 4| Linearity of the Assay

DNA from two cell lines with known *FLT3* ITD mutations (30 bp and 126 bp, respectively) were serially diluted into background DNA from a wild-type *FLT3* cell line and tested with the *FLT3* ITD MRD Assay. Input DNA quantity was 700 ng per dilution point. The *FLT3* ITD MRDDAT software was used to analyze the data **(Table 1)**. As shown in **Figure 2**, the linearity of the assay is excellent in the mutation/total reads range of $10^{-1}$ – $10^{-5}$.

**TABLE 1.** LINEARITY OF THE *FLT3* ITD MRD ASSAY

| EXPECTED MUTATION/TOTAL READS | DETECTED | |
|---|---|---|
| | CELL LINE A | CELL LINE B |
| | ITD MUTATION/TOTAL READS | ITD MUTATION/TOTAL READS |
| $10^{-1}$ | 7.40E-02 | 9.07E-02 |
| | 7.61E-02 | 9.81E-02 |
| | 7.66E-02 | 9.60E-02 |
| | 7.61E-02 | 7.96E-02 |
| $10^{-2}$ | 7.55E-03 | 1.45E-02 |
| | 7.74E-03 | 1.49E-02 |
| | 7.36E-03 | 1.40E-02 |
| | 7.44E-03 | 1.56E-02 |
| $10^{-3}$ | 7.46E-04 | 1.53E-03 |
| | 9.74E-04 | 1.57E-03 |
| | 8.16E-04 | 1.39E-03 |
| | 7.64E-04 | 1.35E-03 |
| $10^{-4}$ | 9.18E-05 | 1.55E-04 |
| | 8.42E-05 | 1.39E-04 |
| | 5.67E-05 | 1.36E-04 |
| | 1.02E-04 | 1.38E-04 |
| $10^{-5}$ | 5.76E-06 | 1.22E-05 |
| | 1.65E-05 | 2.42E-05 |
| | 0.00E+00 | 6.54E-06 |
| | 0.00E+00 | 7.85E-06 |

## 5| Precision and Reproducibility

The precision and reproducibility of the *FLT3* ITD MRD Assay was demonstrated by testing DNA from two cell lines and a clinical sample that had mutation to total read ratios in the range of $10^{-3}$ – $10^{-5}$. Input DNA quantity was 700 ng per sample. The data was generated by two operators each constructing two libraries, and run on two different instruments. The results show excellent precision (data not shown) and reproducibility **(Table 2)**. The coefficients of variation (CVs) are outlined below for samples with mutation/total reads of $10^{-3}$ and $10^{-4}$, for which the 95% confidence level was reached.

**TABLE 2:** REPRODUCIBILITY OF THE *FLT3* ITD MRD ASSAY

| SAMPLE NAME | EXPECTED MUTATION/ TOTAL READS | SAMPLE SIZE | TOTAL CV (%) |
|---|---|---|---|
| CELL LINE A | $10^{-3}$ | 16 | 8.4 |
| | $10^{-4}$ | 32 | 21.8 |
| CELL LINE B | $10^{-3}$ | 16 | 8.6 |
| | $10^{-4}$ | 16 | 24.4 |
| CLINICAL SAMPLE A | $10^{-3}$ | 16 | 6.3 |
| | $10^{-4}$ | 16 | 28.8 |

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