

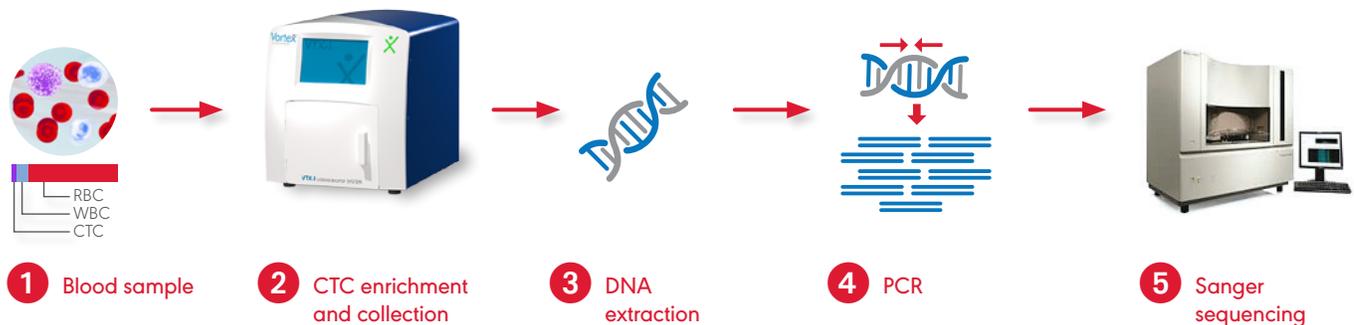
# Detection of KRAS, BRAF, PIK3CA Mutations in Circulating Tumor Cells Using PCR-based Sanger Sequencing

## Overview

Mutation profiling is crucial to guiding treatment decisions. For example, in colorectal cancer (CRC), information on KRAS, BRAF and PIK3CA genotype is extremely valuable in systemic therapy; besides predicting the therapeutic efficiency of anti-EGFR therapy, it can identify patients with poor prognosis.<sup>1,2,3</sup> Mutation profiling is currently completed using tumor biopsy samples, an invasive process that can be limited by small sample size and difficult access to the tumor site. Moreover, single-site tumor biopsies may not be representative of tumor heterogeneity and may fail to reflect the genetic diversity of a patient.

Circulating tumor cells (CTCs) are emerging biomarkers shed by the tumor into the bloodstream and play an important role in cancer metastasis. CTCs may be used in lieu of tumor biopsy for cancer prognosis, disease monitoring and targeted therapeutics because samples can be obtained noninvasively and repeatedly. However, since CTCs are rare, a significant enrichment to high levels of purity is required prior to DNA extraction and Sanger sequencing. The Vortex VTX-1 enriches intact CTCs from whole blood to high levels of purity, allowing for DNA extraction and Sanger sequencing to work with high precision and sensitivity.<sup>1</sup>

## Workflow



DNA from Vortex-enriched CTCs was extracted using QIAamp<sup>®</sup> DNA Micro Kit (QIAGEN) with customized protocol and quantified by qPCR<sup>1,2</sup>. DNA from frozen metastatic liver tissues or primary cancer FFPE tissues was extracted by QIAamp DNA Micro Kit or GeneRead DNA FFPE Kit (QIAGEN) correspondingly and quantified by Qubit<sup>®</sup> 3.0 Fluorometer (Thermo Fisher). The extracted DNA was subjected to PCR amplification using AmpliTaq Gold<sup>®</sup> 360 PCR Master Mix (Thermo Fisher) and corresponding primers

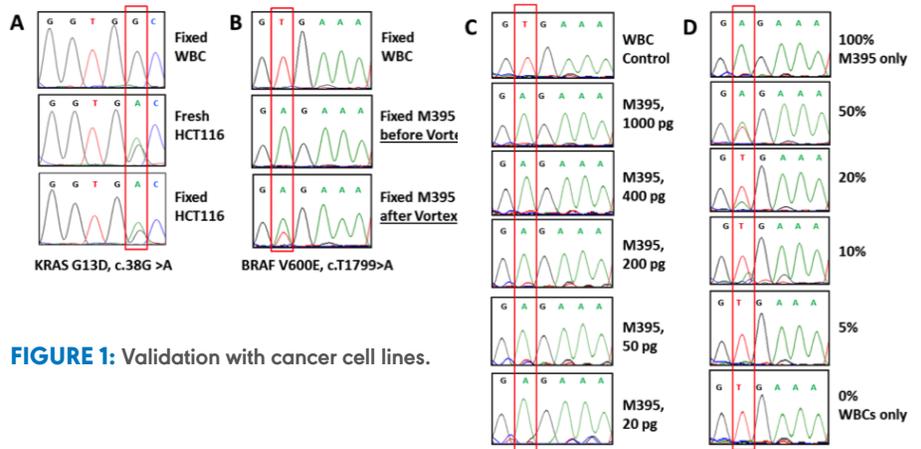
at 95°C for 5 min, 35 cycles of 94°C for 30 sec, 58°C for 45 sec, 72°C for 45 sec and finally 72°C for 10 min for the final extension. The PCR products were then qualified and quantified by E-Gel<sup>®</sup> Electrophoresis System (Thermo Fisher) and Qubit 3.0 before purification by QIAquick<sup>®</sup> PCR Purification Kit (QIAGEN). The purified PCR product was sequenced on a 3730xl DNA Analyzer (Thermo Fisher) and the ABI chromatogram files were analyzed by BioEdit sequence alignment editor.

# Results

## Validation with cancer cell lines

Our workflow and primers were first validated with cancer cell lines: HCT116 (Figure 1A) and M395, fresh or fixed with 4% PFA. Cells can be spiked in healthy blood, processed through Vortex, and DNA mutations detected afterwards (Figure 1B).

The workflow was tested to determine limitations in terms of DNA input and purity. Accurate sequencing results were obtained from DNA input as low as 20 pg (Figure 1C) and DNA purity >10% (Figure 1D).



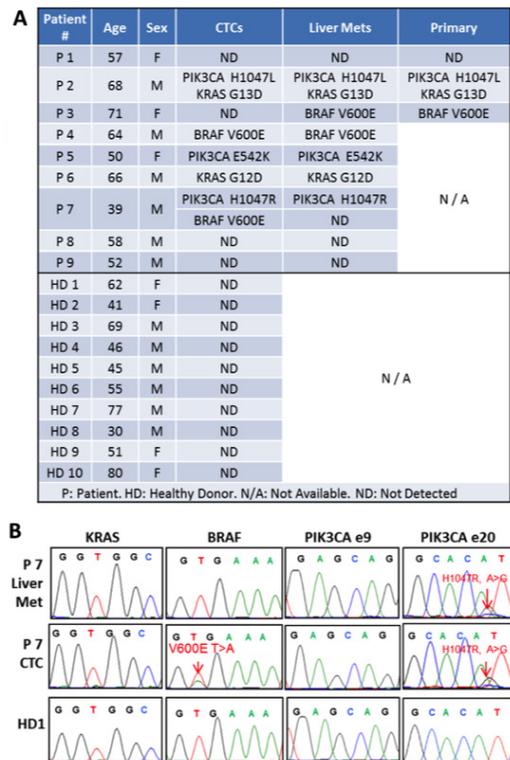
**FIGURE 1:** Validation with cancer cell lines.

## Validation with metastatic colorectal cancer (mCRC) patient samples.

Similar mutations were detected in CTCs, primary tumor and FFPE samples from the same patient (Figure 2A, B).<sup>4</sup> No mutation was detected in age-matched healthy donors (N=10).

## Conclusions

- CTCs collected with Vortex technology can be seamlessly integrated within a genomic workflow, i.e. DNA extraction, PCR and Sanger sequencing.<sup>4</sup> This protocol can be adapted to other gene mutations as well.
- PCR-based Sanger sequencing does not require preliminary whole genome amplification (WGA), which makes it fast, simple, cheap, less biased and usable on both fresh and fixed cells. The high purity of CTCs achieved by enrichment using the Vortex VTX-1 system makes it ideal for Sanger sequencing assays.
- Here we have shown the detection of KRAS, BRAF and PIK3CA mutations on fixed CTCs. Similarly, other PCR-based assays such as qPCR or digital PCR can potentially be applied to these CTCs.



**FIGURE 2:** Validation with metastatic colorectal cancer (mCRC) patient samples.

## References

1. Vortex Application Protocols: DE001 [DNA extraction from rare cells], DQ001 [DNA quantification by qPCR], DS001 [PCR-based Sanger sequencing].
2. Applied Biosystems: application note: KRAS variant identification. [https://tools.thermofisher.com/content/sfs/brochures/cms\\_072026.pdf](https://tools.thermofisher.com/content/sfs/brochures/cms_072026.pdf).
3. Karapetis CS, Jonker D, Daneshmand M, et al. NCIC Clinical Trials Group and the Australasian Gastro-Intestinal Trials Group. PIK3CA, BRAF, and PTEN status and benefit from cetuximab in the treatment of advanced colorectal cancer—results from NCIC CTG/AGITG CO.17. *Clin Cancer Res* 2014 Feb;20(3):744-753.
4. Kiddes-Sigal E, Liu H, Triboulet M, et al. Enumeration and mutational profiling of CTCs, and comparison to ctDNA and colorectal cancer liver metastases. ISMRC 2016 poster. AACR 2016 poster.