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Digital Sample Enrichment and RT-castPCR Detection for Direct Molecular Characterization and Enumeration of Circulating Tumor Cells (CTC)

David Xingfei Deng, Scott Sproul, Yun Bao, Yu Wang, Fawn Wang, David Merrill, Pius Brzoska, and Caifu Chen
Life Technologies, 850 Lincoln Centre Dr. Foster City, CA94404, USA

life technologies™

ABSTRACT

Enumeration and molecular characterization of circulating tumor cells (CTCs) promise to be valuable for cancer diagnosis, survival prognosis, and treatment guidance. However, current methods require extensive enrichment process before analyzing rare CTCs in human blood. We reported here a new approach for direct CTC detection in whole blood samples by using digital sample enrichment and competitive allele-specific TaqMan PCR (castPCR) for rare mutations and RT-qPCR for cell type-specific genes. Whole blood samples from lung cancer patients or normal individuals with spiked-in known lung cancer cell lines were partitioned in aliquots of 2.5 - 50 µL onto 96- or 384-well plates, such that each well contained either one cancer cell or none in the presence of $2x10^4$ - $4x10^5$ normal white blood cells and 10^7 - $2x10^8$ red blood cells. The sample partition process resulted in a digital enrichment of 20 – 400 folds (the relative ratio of CTC to normal cells) in a CTC-positive well. castPCR clearly identified known mutations and CK19 in spiked-in samples of ~10 - 30 cells per mL whole blood, but there was no positive well in the absence of spiked-in cells. Furthermore, cell type specific markers (CK19) and known EGFR mutations were identified in the same sample wells, indicating that identified mutation was specifically derived from cancer cells. In two blood samples from lung cancer patients, EGFR mutation (p.L858R) was detected in all samples. Approximately, 50% of circulating lung tumor cells in a patient with positive EGFR p.L858R mutation had also positive EGFR p.T790M mutation, an inducible drug-resistant CTC marker. For those samples with negative detection of EGFR mutation, corresponding wild type sequences were detected in all sample wells. In conclusion, our data suggest that combination of digital sample enrichment with castPCR and RT-qPCR could be used to directly enumerate CTCs and detect cancer mutations in whole blood without prior biophysical sample enrichment. This new approach may pave the way for noninvasive CTC monitoring and individualized therapy.

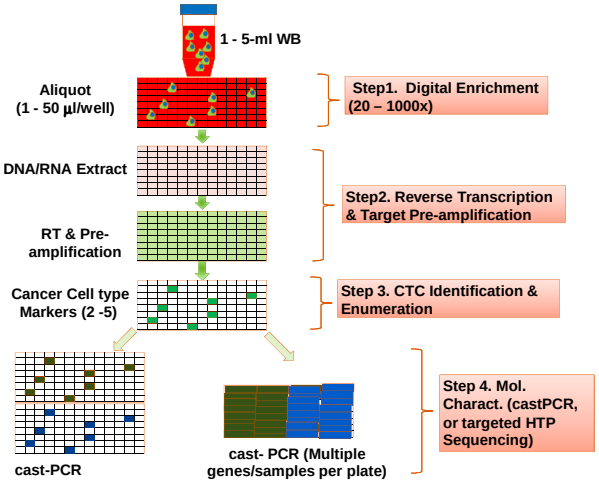
Challenges of CTC Analyses

- **CTCs Are Rare in Blood**
  - ~ 1 cell/mL Blood
  - Huge background cells (~1 in 10^6 WBC)
- **Variable Level of CTC Surface Antigen**
  - Unreliable isolation via cell surface markers
- **Ectopic Expression and Genetic Mutations in "Normal" Blood Cells**
  - Difficult in data interpretation from whole blood

Our New Strategies

- **Sample Partition (Digital Enrichment)**
  - 100 – 1000 x enrichment
  - Independent of cell surface markers
- **High Selective Assays: castPCR**
  - Up to 1 in 10^6 selectivity
- **Mutation Detection from Both RNA and DNA**
  - Multiple copies in a cell
- **Co-relate Cancer cell markers and mutation markers in the same CTC**

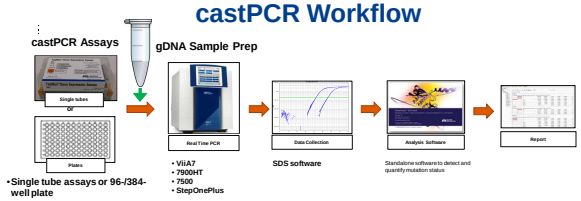
Digital Sample Enrichment and RT-castPCR Detection



- Enrich CTCs with no target cell loss
- Do not depend upon cell surface antigens
- Link mutation markers to cancer cell markers

CastPCR Detection Has High Sensitivity and Specificity

castPCR Workflow

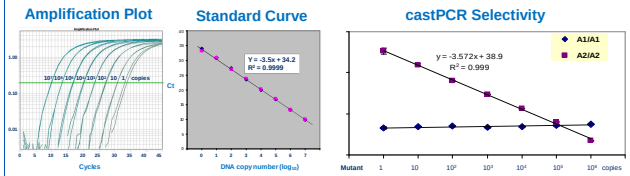


- Single tube assays or 96-/384-well plate
- Assays includes target mutants and wild types
- Reference assays for quantification
- Duplexed with IPC assay for detecting IPC failure

- ViiA7
- 7900HT
- 7500
- StepOnePlus

(2 ½ h)

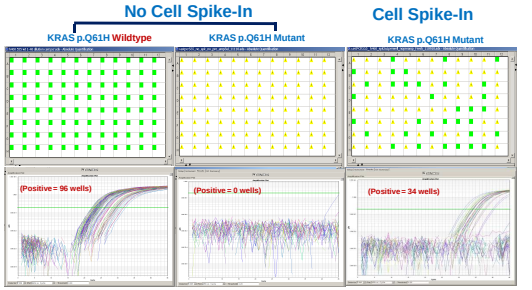
castPCR Sensitivity and Selectivity



- Plasmid DNA copies: 1 – 10 million copies
- Excellent linearity over > 6 logs dynamic ranges

castPCR assays can detect up to 1 copy of mutant DNA in the background of 2.5 µg wild-type genomic DNA

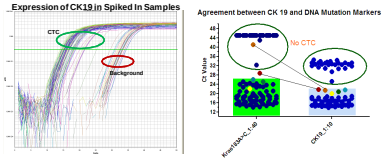
castPCR Detection Accurately Determine Spiked-In Cancer Cells



- Cancer cell line H460 was spiked into normal blood about 35 cells per mL
- Blood sample was aliquoted into 96-well plate in 10 µL per well and castPCR assay was performed

Cell Type Markers (CK19) Co-relate To Mutation Markers

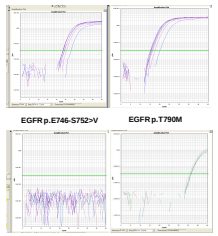
- H460 cell line with known KRAS mutant spiked-in normal blood
- CK19 and KRAS mutant were detected in the same sample wells



CastPCR Detection Accurately Determine CTCs From Lung Cancer

- Blood samples from 5 lung cancer patients (stage >IIIB)
- Blood were aliquoted in 50 µL onto 96-well plate
- Selected some of the CK19 positive wells from each sample for CastPCR EGFR assays

Cosmid ID	12384	6253	6224	6240	12369	Mix
CDS MUT SYNTAX	c.2237 2255>T	c.2155G>T	c.2573T>G	c.2369C>T	c.2240 2254del15	EGFR 19
AA MUT SYNTAX	p.E746 S752>V	p.G719C	p.L858R	p.T790M	p.L747 T751del	12 mutations
Primers over Intron	No	No	No	Yes	Yes	Yes
Positive Mutant CTC	0	32	32	11	0	0
CTC Tested	32	32	32	32	32	32



- CTC detected all lung patient samples tested by:
  - Positive CK19
  - Positive EFGR mutation
- All CTCs with positive CK19 and EFGR mutations (p.L858R & p.G719C)
  - ~ One third of CTC had p.T790M mutation

Summary

We present results for direct CTC molecular characterization and enumeration in blood:

- Up to 1000 fold sample enrichment by simple partition
- castRT-qPCR assays detecting cancer markers and mutations from the same CTCs
- New approach can be applied to CTCs of all cancer types for direct molecular characterization and enumeration