Mutational Analysis in Circulating Tumor Cells (CTC):ScreenCell MB Filtration Unit and ICE COLD-PCR

Katherine A. Richardson[1], Grant Wu[1], Yanan Kuang[2], Lilin Wang[2], Benjamin Legendre, Jr.[1], Rui Lin[1], Yanggu Shi[1], Phil Eastlake[1], Yvon Cayre[3], Pasi Janne[2], Robert Distel[2]. [1]Transgenomic, Inc, Omaha, NE; [2]Dana Farber Cancer Institute, Boston, MA; [3]ScreenCell, Paris, France
Email: info@transgenomic.com

ACR
Poster #2090

ABSTRACT

The aim of this study was to validate the use of the ScreenCell® MB Filtration Units for detection of mutations in Circulating Tumor Cells. This device allows physicians to easily isolate any CTCs using a simple blood draw which can be performed at a local doctor's office. This is especially important when trying to analyze CTCs in elderly patient populations and in regions where the large medical centers are far away. Once isolated, the filter containing the CTCs can be sent to a laboratory for DNA isolation and the mutation analysis. Mutational analysis using ICE COLD-PCR can then be performed without the use of expensive or specialized equipment. The mutation was characterized using standard Sanger DNA sequencing with a detection limit of 0.5 - 0.1% mutant sequence in the wild-type background.

Results will be presented from a mock experiment where PC9 cells (EGFR exon 19 deletion: p.E746_A750del) or H1975 cells (EGFR exon 21: p.L858R) were spiked into blood and then the mock CTCs were collected on the ScreenCell Molecular Filter Device. Following DNA isolation, the mutations were easily detected at the lowest number of cells spiked in to the blood samples (10 cells). Additional data from CTCs collected from NSCLC patients detected the L858R point mutation, an exon 19 E746_A750del mutation, and an exon 19 E746_S752delinsV. Mutations in these same samples could not be detected in DNA sequencing following standard PCR amplification.

These data show that the ScreenCell MB Filtration Unit coupled to mutational analysis using ICE COLD-PCR can be an easy, cost-effective method for collection and analysis of CTCs in patient blood samples. Moreover, the device is simple enough to be used in any doctor's office.

INTRODUCTION

Isolation and characterization of cancer patients' circulating tumor cells (CTC's) will allow physicians to determine the mutation load of the patient; potentially determine which compounds will be most effective in treating the cancer; and allow monitoring and adjusting treatment dependant upon which mutations are present in the metastatic CTCs. In order to benefit cancer patients world-wide, all CTC's (from the most epithelial to the most mesenchymal) must be easily isolated from a simple blood draw and analyzed without the need of the site to have expensive or specialized equipment readily available. The ScreenCell Molecular Biology filtration device uses size-based filtration which allows the non-biased capture of all CTC's present in up to 6 mls of blood (1). The filter containing the captures CTC's can then be sent to any molecular biology facility for mutation testing.

CTC's are in low numbers in the blood and those containing mutations may be a subset of these cells, therefore a sensitive methodology is needed to detect mutations in this population of cells. Transgenomic has obtained both exclusive and non-exclusive licenses for commercializing The Dana Farber's COamplification at Lower Denaturation temperatures (COLD-PCR) technology (2). COLD-PCR is a significant new discovery in deep mutation detection sensitivity. A variation known as ICE COLD-PCR (Improved and Complete Enrichment COLD-PCR), has proven to deliver the highest mutation enrichment rates while detecting all mutations present in the reaction (3). In ICE COLD-PCR, a reference sequence oligonucleotide (RS-oligo) is added to the COLD-PCR reaction. This RS-oligo contains several locked nucleic acid (LNA) bases and is complimentary to one strand of the wild-type sequence, but not to the mutant sequence. Incorporation of LNA bases constrains the RS-oligo in the "A" conformation for Watson-Crick base pairing and causes more rapid and stable hybridization to the complimentary wild-type sequence. RS-oligos containing LNA's generally result in higher Tm's for wild-type:RS-oligo than for mutant:RS-oligo duplexes. This results in preferential amplification of the mutant DNA. (Figure 1) and results in a limit of detection of ≤ 5% with confirmation by Sanger sequencing.

In the initial proof of concept experiments, blood was spiked with 0, 100, 50, or 10 cells which had known EGFR exon 19 deletions and the L858R point mutation. This experiment was performed (a) to determine if the ScreenCell filtration devices could capture mutant-containing cells, and (b) to determine if the ICE COLD-PCR assays could detect the mutations present in a background of wild-type DNA coming from a few lymphocytes which are also collected by the filter. CTC's collected from the blood of three NSCLC cancer patients with tumors responsive to TKI inhibitor therapy and where the genotype of the tumor had been determined, were analyzed using the ICE COLD-PCR assays developed for EGFR exons 19 and 21.

Figure 1: Mutant enrichment by ICE COLD-PCR

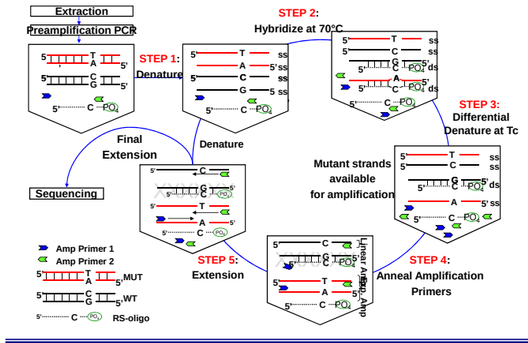


Figure 2: ScreenCell® MB Filtration Device

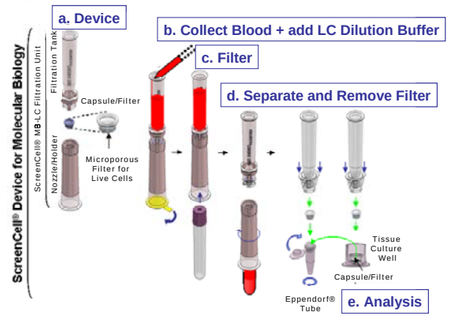
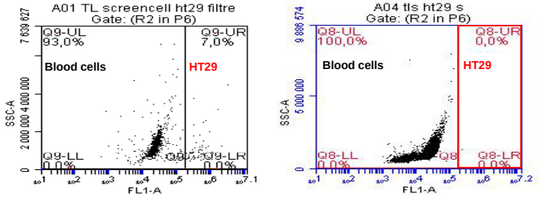


Figure 3: FACS Detection of Mock Tumor Cells



FACS Detection of GFP+ HT29 cells on the filter

No GFP+ HT29 cells Detected by FACS in the filtered blood

Figure 4: Recovery of 5 or 2 Cells Spiked into Blood and Recovered on the ScreenCell Filter Devices

	Exp #1		Exp #2		Exp #3		Exp #4		Exp #5	
# Cells Spiked in 1 mL of Blood per Filter	5	2	5	2	5	2	5	2	5	2
Filter 1	4	2	5	1	4	2	5	1	4	2
Filter 2	5	2	5	1	5	0	3	2	5	2
Filter 3	3	0	4	2	5	2	5	1	5	2
Filter 4	5	2	4	2	4	1	5	0	5	2
Filter 5	4	2	5	1	5	2	5	1	5	2
Recovery per Filter	84%	80%	92%	70%	92%	70%	92%	50%	96%	100%

Fig. 4: Overall recovery of 5 cells = 91.2% and the overall recovery of 2 cells = 74%

Figure 5: ICE COLD-PCR Detection of EGFR Mutants in Proof of Concept Experiment

Sample Name	Sequences: ICE COLD-PCR without RS-oligo (PC9 cells)	Sequences: ICE COLD-PCR with RS-oligo (PC9 cells)	Sequences: ICE COLD-PCR without RS-oligo (H1975 cells)	Sequences: ICE COLD-PCR with RS-oligo (H1975 cells)
1% Mutant Control	NVD	E746_A750del (c.2235-2249del15)	NVD	L858R
0 Mutant Cells	NVD	NVD	NVD	NVD
10 Mutant Cells	NVD	E746_A750del (c.2235-2249del15)	NVD	L858R

Fig. 5: ICE COLD-PCR detection of mutations in EGFR ex 19 or 21 from 10 spiked PC9 or H1975 cells. If the RS-oligo is not present in the ICE COLD-PCR reaction, the mutations cannot be detected.

RESULTS

ScreenCell Filtration devices were tested at ScreenCell to determine if mock CTC's pass through the filter into the waste. HT29 cells containing a GFP+ label were mixed with blood and then the blood was filtered. The results (Figure 3) indicate that the CTC's are trapped by the filter and do not pass through into the waste. An additional experiment performed at ScreenCell, indicated that the overall recovery by the filter of 5 spiked cells was 91.2% and that of 2 spiked cells was 74% (Figure 4).

In cell line Proof of Concept experiments the EGFR exon 19 deletion and the L858R mutations were only detected when the sample was analyzed using ICE COLD-PCR assays containing the RS-oligo. Mutations were detected in the spiked blood samples containing 100 (not shown), 50 (not shown) or 10 cells and not in the DNA isolated from the filter with 0 cells spiked into the blood. The representative sequencing electropherograms are shown in Figure 5.

The CTC's from 3 NSCLC responsive patients' blood were collected using the ScreenCell MB filtration device at the Dana Farber Cancer Institute. The DNA was isolated sent to Transgenomic for ICE COLD-PCR analysis of EGFR exons 19 and 21. The sequencing electropherograms are shown in Figures 6- 8. In EGFR exon 19, the E746_A750 deletion was detected in one patient and a complex deletion (E746_S752delinsV) was detected in the other patient. The third patient contained the L858R and not the L861Q mutation in exon 21.

Figure 6: ICE COLD-PCR Detection of an EGFR exon 19 Deletion in DNA Isolated from CTC's collected with the ScreenCell MB Device from the Peripheral Blood of a NSCLC Patient

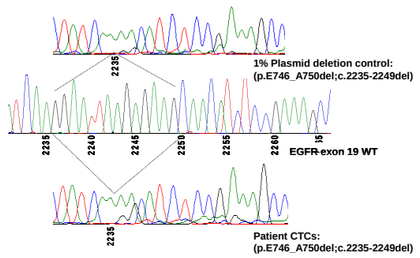


Figure 7: ICE COLD-PCR Detection of a Complex EGFR exon 19 Deletion in DNA Isolated from CTC's collected with the ScreenCell MB Device from the Peripheral Blood of a NSCLC Patient

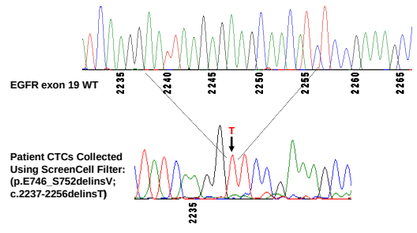
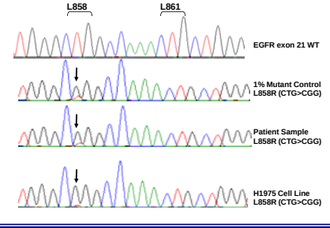


Figure 8: ICE COLD-PCR Detection of the EGFR exon 21 L858R Point Mutation in DNA Isolated from CTC's collected with the ScreenCell MB Device from the Peripheral Blood of a NSCLC Patient



MATERIALS AND METHODS

Samples: The filtration devices were provided by ScreenCell (Paris, FR). The retention and recovery of CTC's by the filter was performed at ScreenCell. ScreenCell filters with 0, 100, 50, or 10 mock CTC cells for the proof of Concept experiment were provided by the Dana Farber Cancer Institute (Voston, MA). NSCLC patient DNA's were isolated from the CTC cells collected by the ScreenCell MB device and purified at the Dana Farber Cancer Institute. The development of the ICE COLD-PCR assays for EGFR exons 19 and 21 was done by Transgenomic, (Omaha, NE). The ICE COLD-PCR analysis and sequencing of the DNAs from the CTC's was performed by Transgenomic.

Retention of CTC's by the filters: 3000 GFP+ labeled HT29 cells were mixed with 3.0 mL of blood. This blood was passed through the ScreenCell device to collect the HT29 cells. FACS analysis was performed on the filter and on the blood collected after filtering. The HT29 cells are present on the filter and not in the waste.

Recovery of CTC's by the filters: Two or five cells were spiked into 1.0 mL of blood and the CTC's were recovered by passing the blood through the filtration device and the number of cells on the filter was counted. Five sets of experiments using 5 different filtration devices were performed for the 5 cell and the 2 cell spiking experiments.

Proof of Concept: Zero, 10, 50, or 100 PC9 cells (E746_A750del) or H1975 dells (L858R) were spiked into blood and then collected using the ScreenCell MB filters. The DNA was isolated using QiaAmp DNA micro kit and subjected to ICE COLD-PCR followed by standard Sanger sequencing.

NSCLC patient CTC's: CTC's were collected at the Dana Farber Cancer Institute from 3 NSCLC patients where previous analysis indicated that 2 of the tumors contained EGFR exon 19 deletions and one contained an exon 21 L858R point mutation. The DNA from the CTC's along with the DNA from control cell lines (data not shown) was isolated and sent to Transgenomic as blinded samples.

CONCLUSIONS

- The ScreenCell Device is simple to use and cost effective
- Can be used in physician's offices world-wide for isolation of CTC's
- All CTC's in the blood are isolated using this device
- DNA from as few as 10 cells is sufficient for mutation detection (fewer than 10 cells have not been tested at this time)
- ICE COLD-PCR can be used to detect low level mutations in the DNA isolated from CTC's collected from 6 mL of blood.
- ICE COLD-PCR can be used to detect mutations in the EGFR gene in DNA isolated from CTC's collected with the ScreenCell MB Device from peripheral blood of NSCLC patients
  - The mutations detected in the CTC's were the same as those found in the tumors
- Expansion studies using CTC's collected by the ScreenCell Device coupled with mutation detection using ICE COLD-PCR are ongoing.

REFERENCES

1. Desittier, I., Guerrouahen, B.S., Benali-Furet, N., Wechsler, J., Jänne, P.A., Kuang, Y., Yanagita, M., Wang, L., Berkowitz, J.A., Distel, R.J., and Cayre, Y.E. (2011). Rapid Isolation by Size and Characterization of Rare Circulating Tumor Cells. Anticancer Res. 31:427-442.
2. Li J, Wang L, Mamon H, Kulke MH, Berbeco R, Makrigiorgos GM. Replacing PCR with COLD-PCR enriches variant DNA sequences and redefines the sensitivity of genetic testing. Nat. Med. 2008;14:579-584.
3. Milbury CA, Li J, Makrigiorgos GM. Ice-COLD-PCR enables rapid amplification and robust enrichment for low-abundance unknown DNA mutations. Nucleic Acids Res. 2010 Oct 11 . [Epub ahead of print].