Single Cell Analysis of Viral Copy Number

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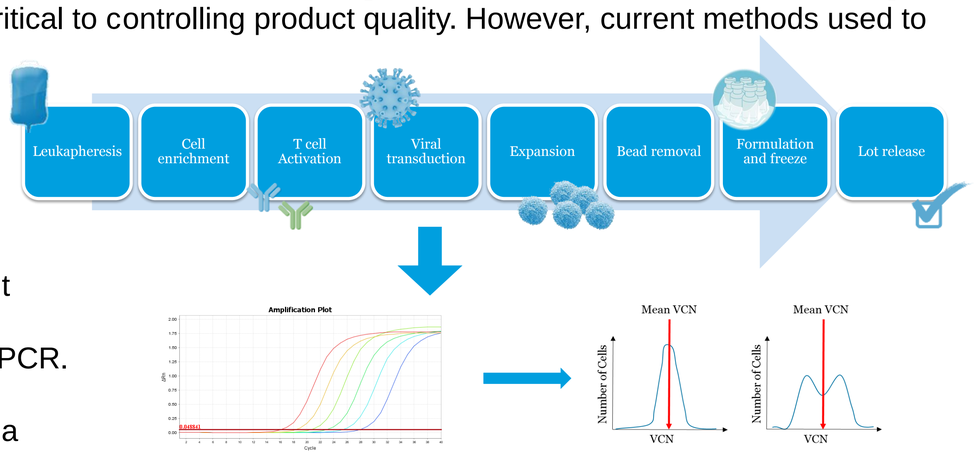
Abstract

Gene modified T-Cell immunotherapies are increasingly being used to treat clinically challenging disease such as haematological cancers. These therapies are often targeted to specific cancers using gene modified T-Cell receptors (TCR) or chimeric antigen receptors (CAR) that are transduced into the T-cells using retroviral or lentiviral vectors. Ensuring consistency during the transduction process through controlled delivery and integration of the viral vectors is critical to controlling product quality. However, current methods used to determine the viral copy number (VCN) in transduced cells involve the analysis of viral sequences from a pool of cells. While this gives an estimate of the VCN within a given population it does not account for cell to cell variation.

To address this:

- We have developed a single cell capture, processing and analysis approach to measure viral copy number integration in a human T-cell immunotherapy model.
- Our approach uses the microfluidic C1 automated capture system from Fluidigm combined with high content screening to isolate, identify and phenotype individual cells.
- Genomic DNA from selected cells is then isolated on the microfluidic chip and prepared for analysis using qPCR.
- By using specially designed primers targeting 2 viral sequences we have been able to successfully detect lentiviral integrations which can be compared to a copy number reference sequence to quantify the VCN at a single cell resolution.

This new characterisation approach offers the potential for increased control over the transduction process, improving a critical step in the immunotherapy manufacture process.



Results

Primer and Chemistry Optimisation

Two approaches were used to optimise viral specific (VS1 and VS2) and copy number reference (CN Ref) primers on pooled cells. Firstly, qPCR was performed on different concentrations of genomic DNA (gDNA) ranging from 1ng (equivalent to 151 cells) to 0.01ng (equivalent to 1.5 cells) using viral targeting primers and Evagreen chemistry (Figure 1) Amplification in fluorescence was detected in transduced cells at concentrations as low as 0.01ng. However, amplification was also seen in non-transduced cells and the melting curve revealed multiple PCR products in both transduced and non-transduced cells. This suggests the presence of non-specific binding.

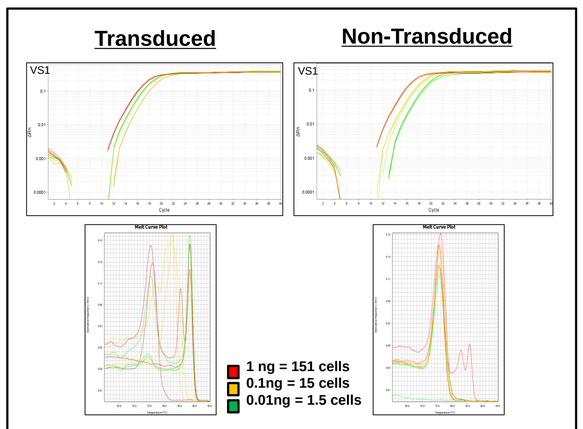


Figure 1: qPCR of viral transduced and non-transduced using viral specific primers (VS1) and Evagreen chemistry.

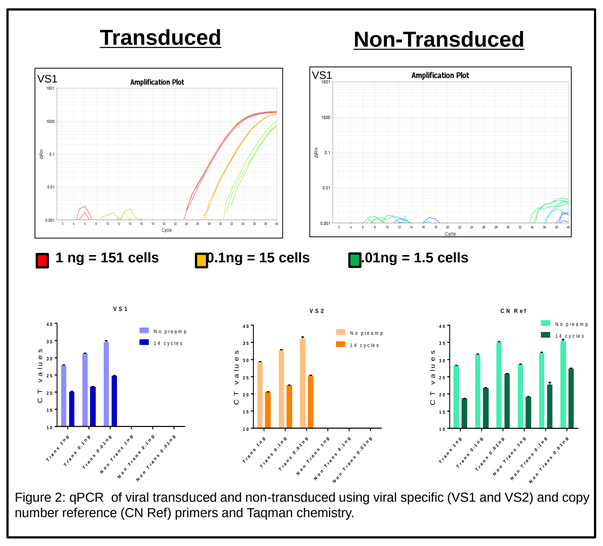


Figure 2: qPCR of viral transduced and non-transduced using viral specific (VS1 and VS2) and copy number reference (CN Ref) primers and Taqman chemistry.

Secondly, qPCR was performed using VS1, VS2 and CN Ref primers with Taqman probes on a range of gDNA concentrations (Figure 2). Viral specific sequences were detected along with the copy number reference sequence in gDNA concentrations as low as 0.01ng (equivalent to 1.5 cells). Ct values of 34-36 for all primer pairs observed at 0.01ng DNA indicated that pre-amplification (pre-amp) cycles are required to reliably detect gDNA from a single cell. Ct values were reduced with 14 pre-amp cycles to 24-27 - such values indicate a likelihood of detecting these specific sequences in a single cell. These pre-amp cycles were used in single cell qPCR experiments.

Single Cell Capture and Imaging

PBMCs were thawed and single cells isolated using the Open App Gene Expression integrated fluidic circuit (Single cell IFC) (Figure 3). Individual capture chambers were imaged using the IN Cell high throughput imager to assess viral induced fluorescence (GFP) and viability (live stain – Calcein Blue, dead stain – Ethidium Homodimer-1). Optimisation of this workflow achieved an ~80% single Cell capture rate. Cell lysis and pre-amp were performed in the single cell IFC using the C1 (Fluidigm). Selected cells were analysed using the FlexSix Chip (qPCR) or 12.765 dPCR chip (digital PCR) on the BioMark (Fluidigm).

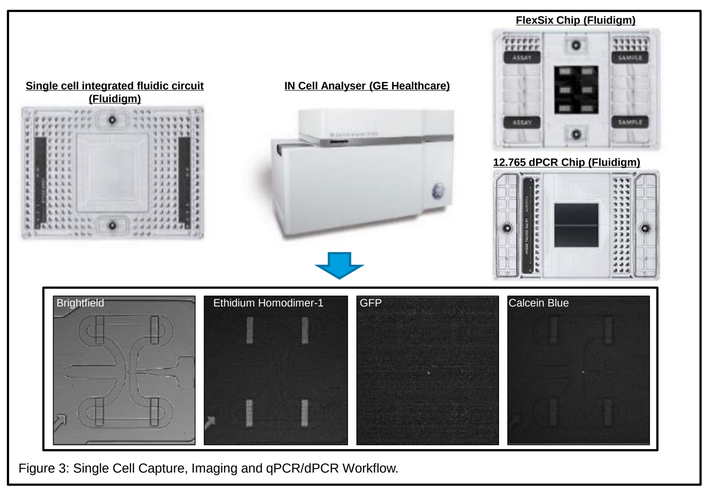


Figure 3: Single Cell Capture, Imaging and qPCR/dPCR Workflow.

Single Cell qPCR

qPCR performed on live single cells and pairs of cells, expressing GFP, revealed the expression of 2 viral sequences (VS1 and VS2) and the detection of the copy number reference sequence (CN Ref) (Figure 4). Non-transduced cells and wells that contained no cells were used as negative controls.

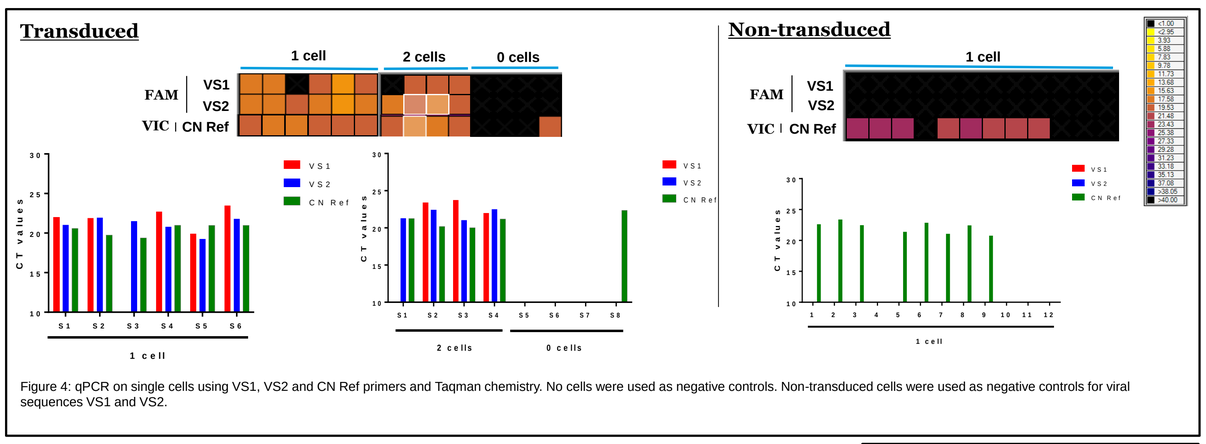


Figure 4: qPCR on single cells using VS1, VS2 and CN Ref primers and Taqman chemistry. No cells were used as negative controls. Non-transduced cells were used as negative controls for viral sequences VS1 and VS2.

The difference in CN Ref Ct values between single and pairs of cells were non significant (Figure 5). Therefore to confidently detect single cells an alternative absolute quantification method was used – digital PCR (dPCR).

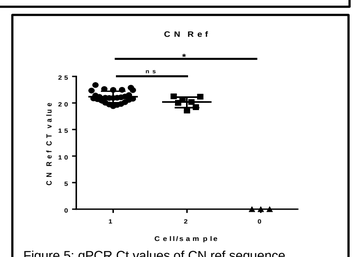


Figure 5: qPCR Ct values of CN ref sequence comparing 0, 1 and 2 cells. Significance was calculated to single cells. * = P<0.05, ns = not significant

Single Cell Digital PCR

Absolute quantification provided by dPCR has the capability to distinguish small changes in copy numbers. Single cell pre-amp products were diluted to levels that produced 50-80% positive dPCR partitions. This level of positive/negative partitions provides the most accurate quantification (Figure 6). The calculated CN Ref copy number showed quantifiable differences between single cells and pairs of cells. However, the quantified copy number was half that of the expected CN Ref value. Consistent copies can be quantified across multiple pre-amp cycles (14-18) suggesting reproducible results can be obtained from single cells using dPCR.

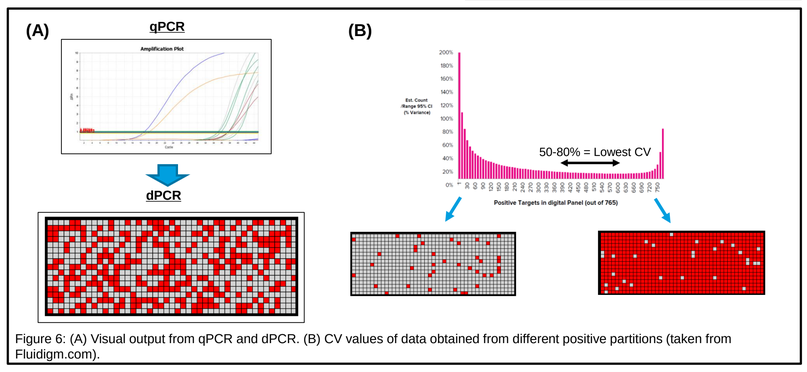


Figure 6: (A) Visual output from qPCR and dPCR. (B) CV values of data obtained from different positive partitions (taken from Fluidigm.com).

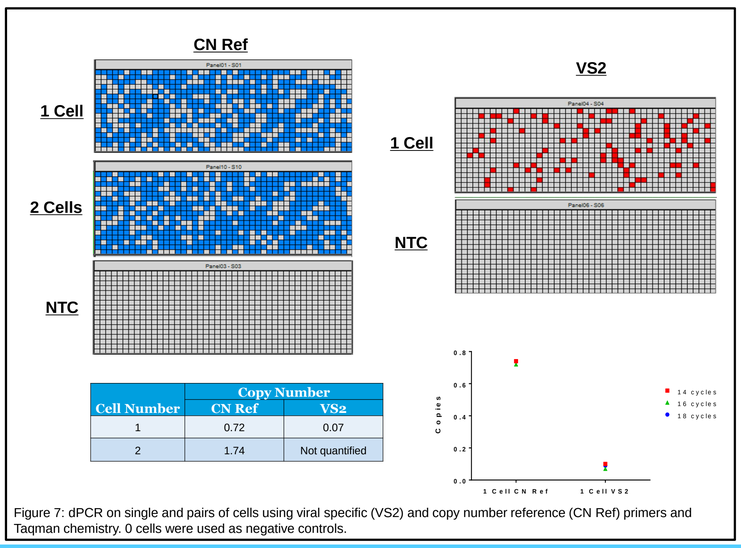


Figure 7: dPCR on single and pairs of cells using viral specific (VS2) and copy number reference (CN Ref) primers and Taqman chemistry. 0 cells were used as negative controls.

Conclusion and Future Challenges

Integrated viral sequences can be detected using both qPCR and dPCR, with the latter having the resolution to show differences between single cells and pairs of cells. However, challenges still remain in accurately quantifying viral copy number. Further research in this area including the use of standard material and the reduction of pre-amp inefficiency via alternative methods including quasilinear PCR, aims to address these challenges. This improved approach to viral copy number quantification number has the potential to increase the control over the transduction process, improving a critical step in the immunotherapy manufacture workflow.

We work with Innovate UK

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