Ultra-Sensitive Duplex Sequencing for Tracking of Allogeneic Cell Therapies

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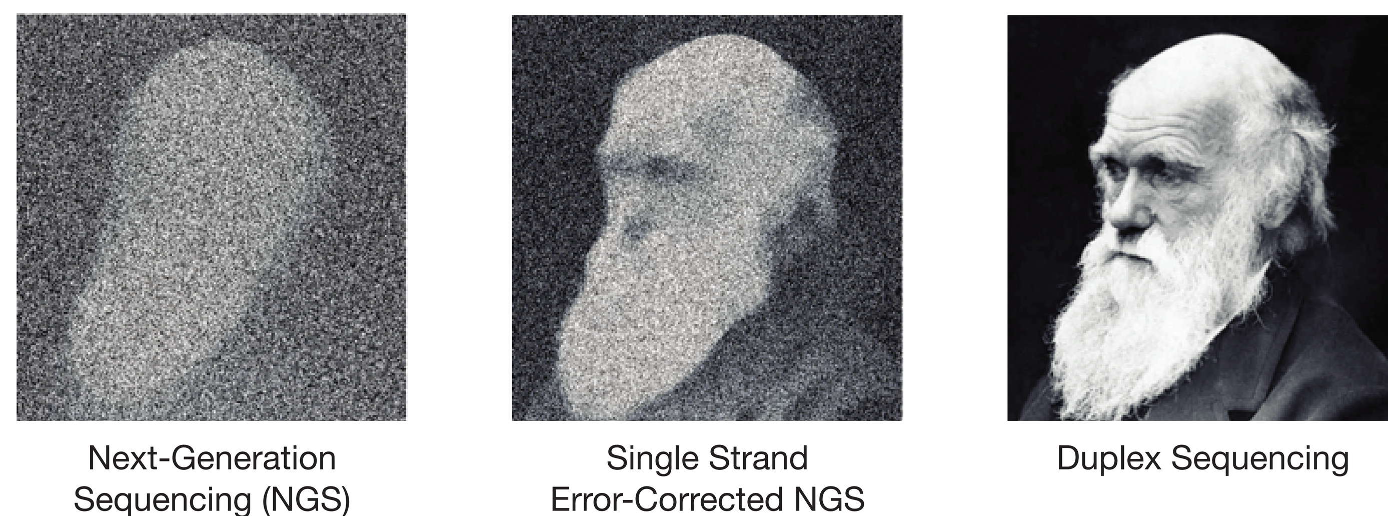
Introduction

Cellular therapies, particularly chimeric antigen receptor T-cells (CAR), are an important emerging treatment modality in oncology. Allogeneic CARs have logistical advantages over autologous ones and are advancing quickly, but monitoring the persistence of the cells during treatment remains a challenge. Current techniques, including droplet digital PCR, are often insufficiently sensitive to detect CARs more than a few days post-infusion and can target only a few alleles. A more sensitive and widely applicable assay would help accelerate development of these treatments.

Duplex Sequencing (DS) compares both strands of each original DNA molecule to eliminate technical errors and achieve extreme accuracy and sensitivity, with an error rate $<10^{-7}$. We designed a hybrid capture panel targeting 277 single nucleotide polymorphisms (SNPs) and one gene knockout target to distinguish donor vs. recipient cells and characterize mutations at the knockout locus with DS. CAR detection power increases linearly with each informative locus because a donor-specific allele at any informative site contributes to a cell population's detection. Therefore, for low input samples, power can be increased by analyzing additional SNPs.

We first prepared synthetic *in vitro* serial dilutions of CAR "donor" DNA into whole blood "recipient" DNA to assess technical performance of the assay. We then performed DS with longitudinal DNA samples from 3 patients receiving an allogeneic CAR therapy. Samples were drawn from pre-infusion up to 1-6 months post-infusion. DS data from pure CAR samples also provided a detailed view of insertions and deletions resulting from CRISPR editing.

Sequencing Errors Obscure Truth

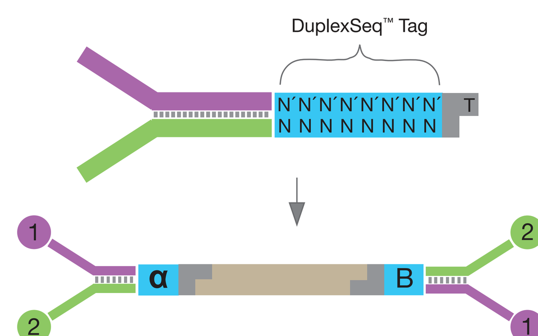


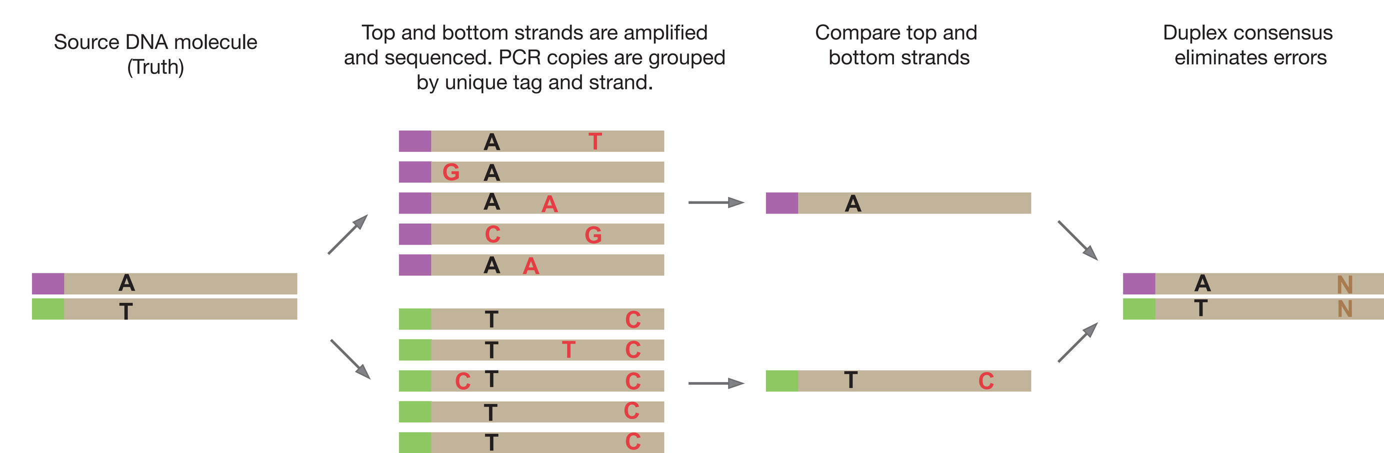
Next-Generation Sequencing (NGS) | Single Strand Error-Corrected NGS | Duplex Sequencing

TwinStrand Duplex Sequencing™ Technology

A DuplexSeq™ Adapter has:

1. Identical (or relatable) degenerate tags in each strand.
2. An asymmetry allowing independent strand identification.





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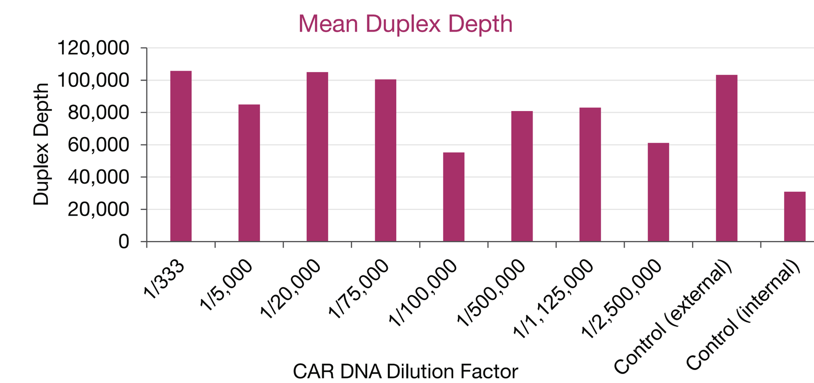
Identify Informative Alleles

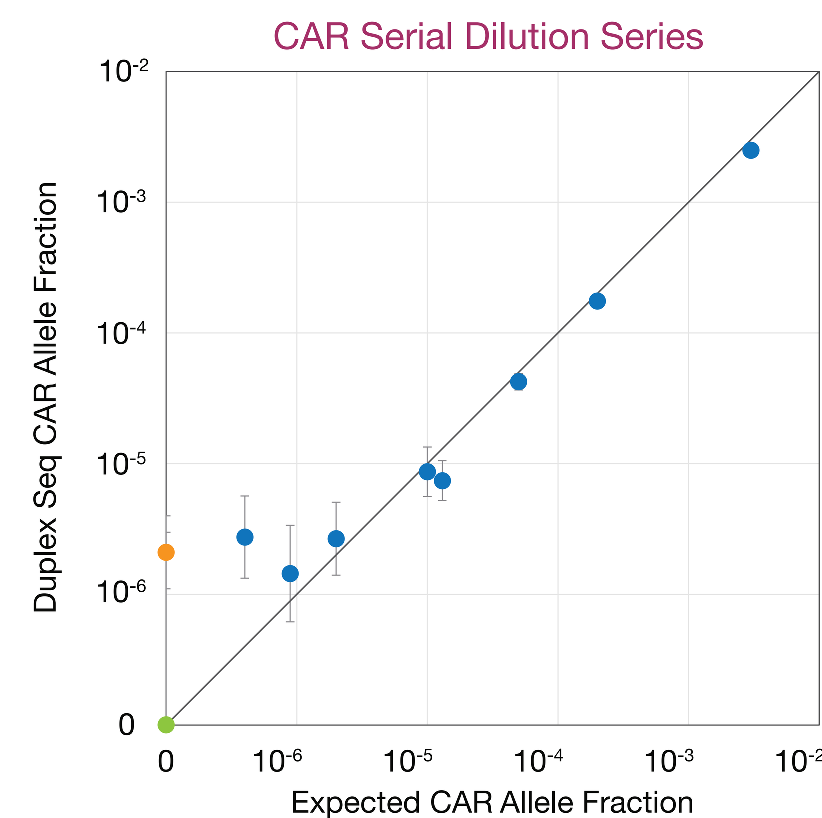
Recipient Genotype		CAR T-Cell Genotype		CAR Genotype Informative?
Homozygous Ref.	A/A	Heterozygous	A/T	YES
Homozygous Ref.	A/A	Homozygous Alt.	T/T	YES
Homozygous Alt.	T/T	Homozygous Ref.	A/A	YES
Homozygous Alt.	T/T	Heterozygous	A/T	no
Homozygous Alt.	T/T	Homozygous Alt.	T/T	no
Heterozygous	A/T	Homozygous Ref.	A/A	no
Heterozygous	A/T	Heterozygous	A/T	no
Heterozygous	A/T	Homozygous Alt.	T/T	no

Where possible, pure CAR T-cell (CAR) donor and patient or diluent "recipient" DNA samples were Duplex Sequenced using a hybrid capture panel targeting 277 single nucleotide polymorphisms (SNPs). Heterozygous or homozygous alleles that uniquely identify the CAR donor were noted. In this example, "A" represents the reference allele and "T" represents the alternate allele.

CAR Allele Detection to 1/1,000,000



CAR DNA was serially diluted into control DNA for expected allelic fractions from 1/333-1/2,500,000. Samples were Duplex Sequenced to 30,901x-105,723x mean Duplex depth, which represents the true molecular depth of original DNA molecules sequenced.

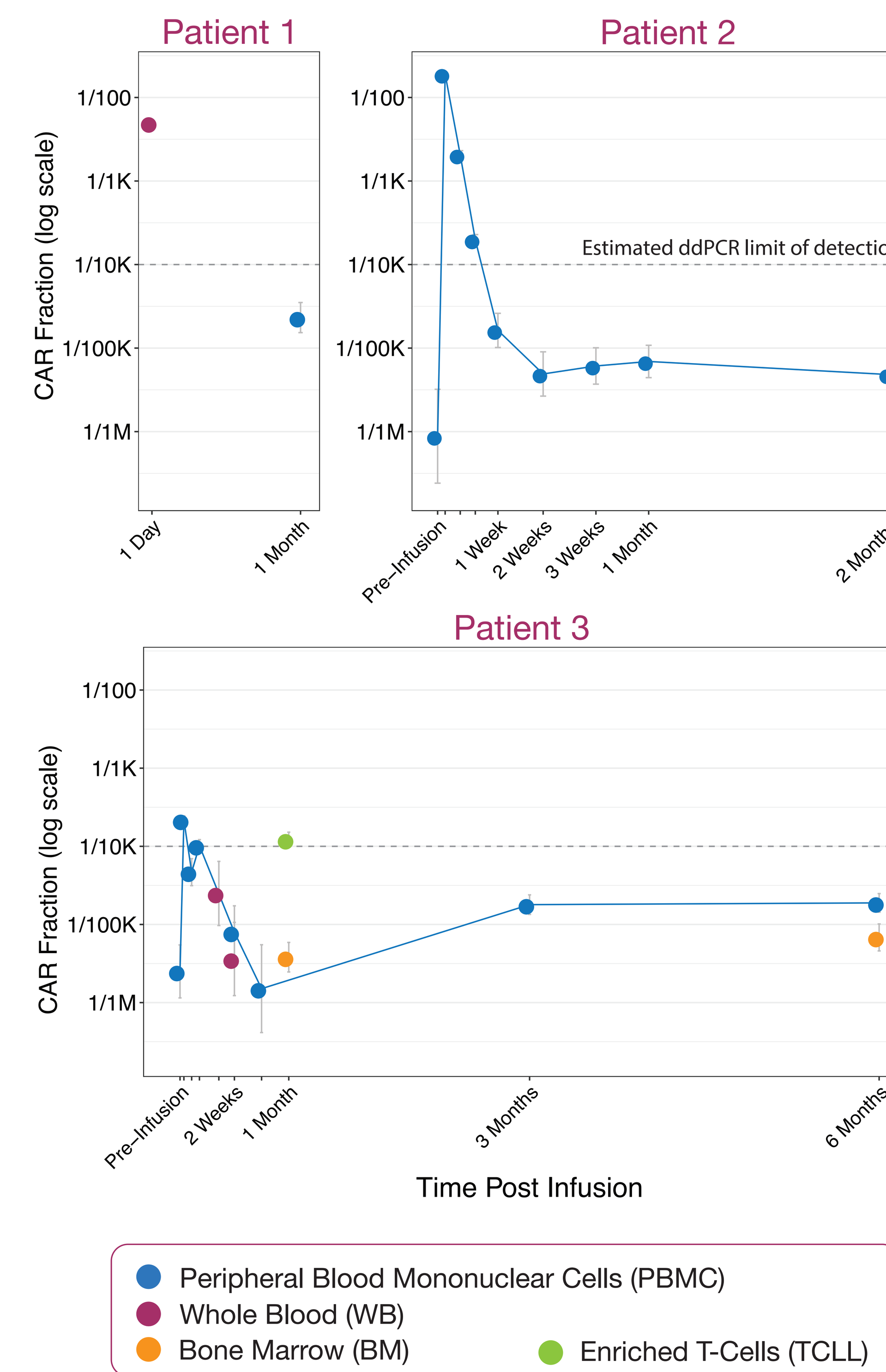


77 informative SNPs were identified, and the overall proportion of CAR alleles per dilution was plotted. The sum of molecular depths at all informative sites serves as the denominator for allele frequency calculations, with heterozygous sites adjusted by a factor of 0.5. Blue dots indicate DNA mixtures. The orange dot represents pure Control DNA that had been handled in an external lab. The green dot represents pure Control DNA that never left TwinStrand Biosciences. CAR alleles can be identified near expected frequencies down to 1/1,000,000 before the signal levels off. Similar allelic signal in the external control DNA suggests low level contamination. However, the lack of any CAR allele signal in the internal control indicates sub-1/1,000,000 detection is possible. Error bars throughout the poster represent 95% Wilson binomial confidence intervals.

CAR T-Cell Engraftment Sustained for Months Post-Infusion

Patient	Informative SNPs	Homozygous in CAR-T	Heterozygous in CAR-T
1	76	23	53
2	73	10	63
3	75	15	60

SNP alleles unique to CARs were identified for 3 patients who had received allogeneic infusions. Longitudinal samples drawn up to 6 months post-infusion were Duplex Sequenced to assess persistence of the CARs. 73-76 informative SNPs were identified per donor-recipient pair, with the majority heterozygous. No pre-infusion DNA sample was available for Patient 1, so the day 28 sample was used to genotype homozygous and heterozygous SNPs. No pure CAR sample was available for Patient 2. However, the 10 SNPs detected at approximately 2% and 63 SNPs at 1% 1 day post infusion served to identify CAR specific alleles.



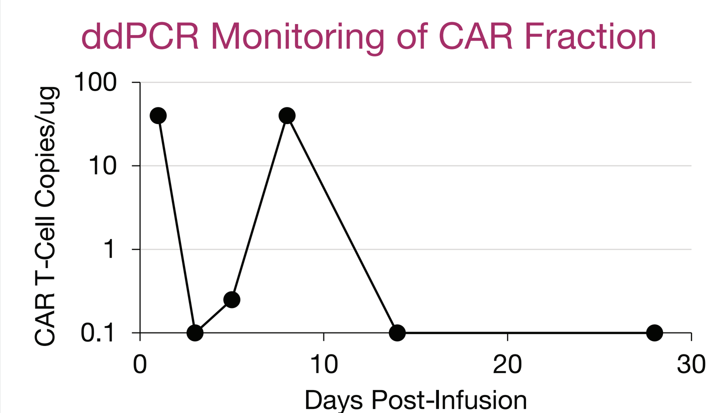
In Patient 1 at Day 1 post-infusion the CAR fraction is nearly 1%. Levels decrease by day 28, yet persistent CARs remain detectable at approximately 1/50,000 ($2.3x10^{-5}$).

Patient 2 was monitored up to 2 months post-infusion. At day 1 the CAR allele fraction is >1%. Levels decrease through day 14, then remain consistent at approximately 1/200,000 ($4.8\text{-}6.9x10^{-6}$) until at least the 2 month time point. The CAR allele signal at all time points is significantly higher than the pre-infusion background of $8.8x10^{-7}$, $p < 0.05$ to $p < 0.001$ by one-sided Fisher test, Benjamini-Hochberg corrected.

T-Cell Enrichment Substantially Increases CAR Allele Signal

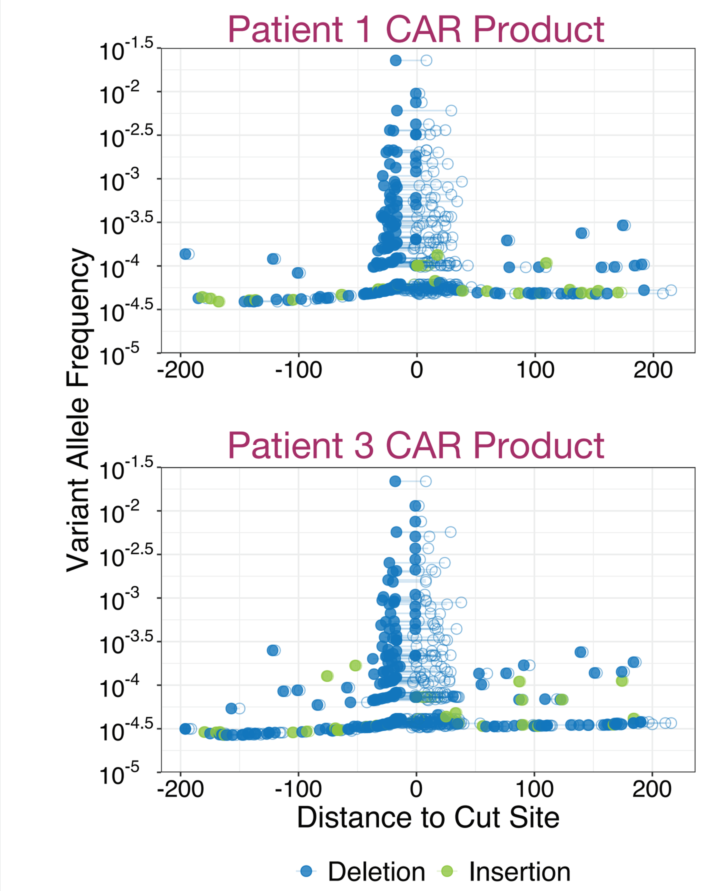
In the lower time course plot to the left, Patient 3 was monitored up to 6 months post-infusion. Anylates include PBMCs, WB, BM and flow-sorted T-cells (TCLL). CAR allele fraction decreased through day 21, but by 3 months it recovered and stabilized at approximately 1/50,000 ($1.8\text{-}1.9x10^{-5}$) in PBMC or 1/150,000 ($6.8x10^{-6}$) in BM. At day 28 the CAR allele fraction in TCLL (green dot) was nearly 2 orders of magnitude greater than in BM, suggesting that flow-sorting T-cells can increase sensitivity for CAR allele detection. CAR allele signal in PBMCs is greater than WB or BM when sampled on the same day. Other than an apparent decrease from day 14-28, CAR allele fraction is significantly greater than pre-infusion signal at least out to 6 months post-infusion ($p < 0.05$ to $p < 0.001$ by one-sided Fisher test, Benjamini-Hochberg corrected).

Limited Sensitivity with ddPCR



Droplet digital PCR (ddPCR) targeting individual SNPs in Patient 5 PBMCs cannot detect CAR alleles above background except at days 1, 5 and 8 post-infusion. This suggests the limit of detection for ddPCR is an allele fraction of approximately 10^{-4}. Therefore even the day 28 TCLL sample plotted to the left may be undetectable with ddPCR.

Quantification of Knockout Mutations



The CARs have had a locus knocked out via CRISPR editing. While knockout alleles could potentially serve as an informative target for CAR detection in patients, using a single locus greatly reduces the power for detection. However, with DS, it is possible to quantify the frequency and type of individual mutations introduced by non-homologous end joining (NHEJ) when resolving CRISPR-mediated breaks. The abundance and proximity to the cut site of deletions (blue) and insertions (green) are depicted for 2 pure CAR products. For deletions, filled points and open circles represent the start and end positions, respectively. Deletions are more common than insertions, and small deletions near the cut site are enriched.

Conclusions

- Duplex Sequencing revealed that infused CAR T-cells persist through the latest time point assayed per patient, which is 1-6 months post-infusion.
- Targeting many informative SNPs increased statistical power for detection. With a single target, sensitivity is limited by the depth at that particular site.
- Duplex Sequencing detected CAR allele fractions down to between 1/100,000-1/1,000,000. That is lower than the Duplex depth at most single informative sites, and 1-2 orders of magnitude below the limit of ddPCR.
- Duplex Sequencing reveals the complex spectrum of individual NHEJ-induced mutations at unprecedented resolution.

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