Combining High-Performance CRISPR Guides with Sensitive, Time-Resolved Cell-Based Assays

Cytotoxicity is not enough, *in vitro* assays must also assess persistence and durability

The great challenge of immune cell-based therapies is to safely engineer cells that have enhanced performance characteristics compared to native immune cells. Although we are still in the early stages of fully realizing this promise, proof of concept has been more than achieved with such hallmarks as the first approved CAR T cell therapies Kymriah and Yescarta.\(^1,2\)

CRISPR gene editing systems have emerged as highly effective surgical tools for modifying genomes in living cells with unprecedented ease. However, to reach their full potential increased activity, stability, and specificity is needed to achieve high fidelity edits with a minimal nuclear footprint. In addition, finding the most effective edits will require live cell analysis tools that deliver both functional relevance and assessment of potency and persistence. Live cell assays that are both quantitative and report out real-time kinetics can achieve these requirements. Based on a wealth of early learnings in immunotherapy, it is well appreciated that cytotoxicity is not enough. Engineered cells must be persistent in a hostile, immunosuppressive, and changing tumor microenvironment throughout the time course of tumor elimination and surveillance to achieve durable results.\(^3\)

To address these challenges, Agilent has brought together an innovative set of solutions that bring greater activity and accuracy to CRISPR editing. These solutions are combined with the ability to assess and validate the results on live-cell platforms that deliver relevant, time-resolved functional data.

**Additional Info**

- CRISPR Solutions
- Real-Time Immune Cell Killing Assays
- Immune Metabolism

Although the future remains bright for CRISPR gene editing, current challenges for wide-spread therapeutic use include better activity, specificity, and tunability for different situations. Guide RNAs that enable targeting of specific genomic loci can be made using enzymes to copy a DNA template into an RNA. However, direct chemical synthesis of guide RNAs such as used for Agilent SureGuide gRNAs\(^4\), has distinct advantages. Chemical synthesis provides a robust method for scalable manufacturing of highly pure sgRNAs, affords unique opportunities for sgRNA design, and precise installation of molecular functionality to augment CRISPR–Cas performance for research, industrial, and therapeutic applications.
Figure 1 shows that chemical modifications added near the ends of guide RNAs can improve the stability and activity of the guide RNAs. In a more recent study, improvements in CRISPR specificity were achieved by incorporating chemical modifications in guide RNAs at specific sites in their DNA recognition sequence and then systematically evaluating their on-target and off-target activities. This novel approach resulted in a dramatic reduction in off-target cleavage activities while maintaining high on-target performance (Figure 2).

Once you have edited your cells how will you assess and validate both the desired and undesired functional consequences? With the emergence of quantitative, time-resolved cell analysis platforms researchers can make more sensitive live-cell measures of function and simultaneously scan the temporal components of an ever-evolving immune response in terms of efficacy and persistence. For example, in a single experiment, one can now test gene edits for both killing efficiencies and serial killing at very low effector to target ratios. The results can then be related to inhibitory effects such as exhaustion, anergy, and other escape mechanisms due to interactions with a constantly evolving tumor and its microenvironment.

A great example of the value of combining functional sensitivity with time resolution was recently published using the Agilent xCELLigence RTCA system. In this study, a CAR T cell strategy to address the heterogeneity of antigen expression within a cancer cell population was investigated. A common observation with adoptive cell therapies is that when cancer cells expressing the targeted antigen are killed off, cells that lack the antigen continue propagating undeterred. To minimize this phenomenon, the authors targeted two tumor cell antigens simultaneously and used xCELLigence to assess both function and persistence. The results shown in Figure 3A enabled the authors to choose the optimal engineering constructs based on a very sensitive quantification of the killing efficacy using low effector to target ratios of various combinations of costimulatory domains with a constant antigen binding domain (N. Ahmed, unpublished results). Simultaneously, they were also able to identify potential escape phenotypes due to the time resolution of the assay.
In Figure 3B, key results are shown for different scenarios where CARs targeting the HER2 and IL13Ra2 antigens were expressed in separate T cells (CARpool), as distinct proteins within the same T cell (biCAR), or as a single fusion protein within T cells (TanCAR). When incubated with glioblastoma target cells each of these CAR T approaches displayed differential killing capacity and kinetics. These nuances in serial killing behavior are readily elucidated by continuous impedance monitoring but would go undetected in traditional end-point assays.

Figure 3. (A) Various combinations of proprietary costimulatory domains with a constant antigen binding domain were quantified for killing efficacy using a low target to effect ratio (1:10) and simultaneously for identification of potential escape phenotypes (pink and orange lines) based on time resolution of the data (N. Ahmed, unpublished results, April 2, 2019). (B) Using xCelligence to monitor the killing of the glioblastoma cell line U373 by CAR-T cells targeting either one or both of the antigens HER2 and IL13Ra2. In the figure legend: U373 = target cell line alone; NT = target cells treated with nontransfected T cells (i.e. not expressing a CAR); IL13Ra2 = target cells treated with T cells expressing a single CAR targeting IL13Ra2; Her2 = target cells treated with T cells expressing a single CAR targeting Her2; see text for descriptions of CARpool, biCAR, and TanCAR. 1:10 E:T ratio. Figure adapted from reference 7.
Another sensitive, time-resolved platform that can deliver rich, functional data is the Agilent Seahorse XF Analyzer. A pivotal study published by the Carl June group showed how different CAR T engineering constructs can have dramatically differential effects on cell fate and function by driving different metabolic programs. It reveals how one might optimize tumor elimination, persistence in the microenvironment, and durability. In Figure 4, a summary of their findings reveals how CAR T cells containing the coreceptor signaling domain, 4-1BB, elicit an aerobic program consistent with central memory formation and persistence. In contrast, engineering with the CD28 coreceptor signaling domain reprograms metabolism towards aerobic glycolysis resulting in enhanced effector memory cell fate. This study was a powerful proof of concept for engineering metabolic traits to establish the right balance of effector and memory cells to address immune cell exhaustion and persistence in hostile tumor microenvironments paving the way for durable memory and immunosurveillance.

References

2. FDA (2017) FDA Approves CAR-T Cell Therapy to Treat Adults with Certain Types of Large B-Cell Lymphoma, [News Release], 18 October.