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Evaluating a Merged Well Analysis Strategy for Validation of a Cellular Kinetics Assay to Quantify an Allogeneic Cell Product in Human Blood by ddPCR

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PURPOSE

Century Therapeutics, Inc. has developed novel product candidates consisting of allogenic induced pluripotent stem cell (*iPSC*)-derived Natural Killer (*iNK*) cells expressing chimeric antigen receptors (*CAR*), which may improve cancer patient outcomes by reducing time-to-treatment and avoiding side effects common in other CAR therapies (*e.g. CAR T*).

Clinical monitoring of expansion and persistence of CAR expressing immune cells is frequently performed via digital droplet PCR (ddPCR), the sensitivity of which may be increased via merged-well analysis.

To characterize this increase in assay sensitivity, we performed parallel validation of a ddPCR cellular pharmacokinetic (PK) assay using both individual-well and merged-well analysis.

OBJECTIVE(S)

Validate the detection & quantification of a CAR iNK-specific target and a genomic DNA loading control (*RPP30*).

Determine the lower limit of quantification (*LLOQ*) and limit of detection (*LOD*) of the duplexed ddPCR cellular PK assay using both individual and merged-well analysis.

METHOD(S)

Master Mix Preparation: A master mix was prepared with Bio-RaddPCR Supermix for Probes, CAR iNK primers and FAM labeled probe, RPP30 primers and HEX labeled probe, and restriction endonuclease. The master mix was aliquoted into a Bio Rad ddPCR *Sample Plate*, and standards and controls were added to appropriate wells.

ddPCR Procedure: The *Sample Plate* was sealed and loaded into a Bio-Rad QX200 automated droplet generator (*AutoDG*), which partitioned the contents of each sample plate well into ~20K nL individual droplets, each containing all the components necessary for a PCR reaction, and transferred the droplets to a ddPCR *Reaction Plate*.

The *Reaction Plate* was loaded into a thermal cycler and endpoint PCR was performed. The *Reaction Plate* was then placed into the Bio-Rad QX200 droplet reader to measure the levels of FAM & HEX fluorescence in each droplet. Droplets in which the target was amplified (*positive*) had higher FAM &/or HEX fluorescence while droplets without amplification (*negative*) had little to no FAM or HEX fluorescence.

Bio-Rad QuantaSoft software used Poisson distribution statistical analysis to determine the absolute quantity of the target in the sample well based on the total number of positive and negative droplets detected. The samples were analyzed with multiple replicates, using both individual- and merged-well analysis in Bio-Rad QuantaSoft Software.

RESULT(S)

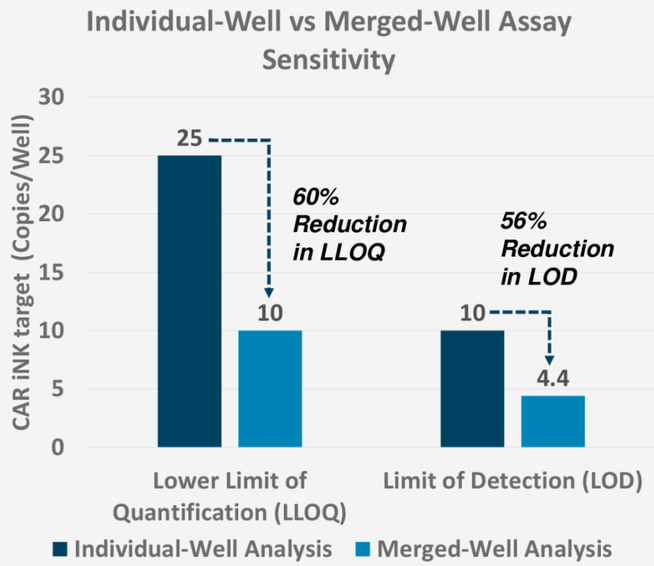
Assay Accuracy, Precision, and Specificity:

Both individual-well and merged-well analysis provide acceptably accurate and precise data (*|RE| and CV ≤ 30% at all points),* linear ($R^2 \geq 0.95$), and specific (*No Template Control, NTC near 0*). Merged-well analysis was performed with merging three wells as one.

Assay Sensitivity:

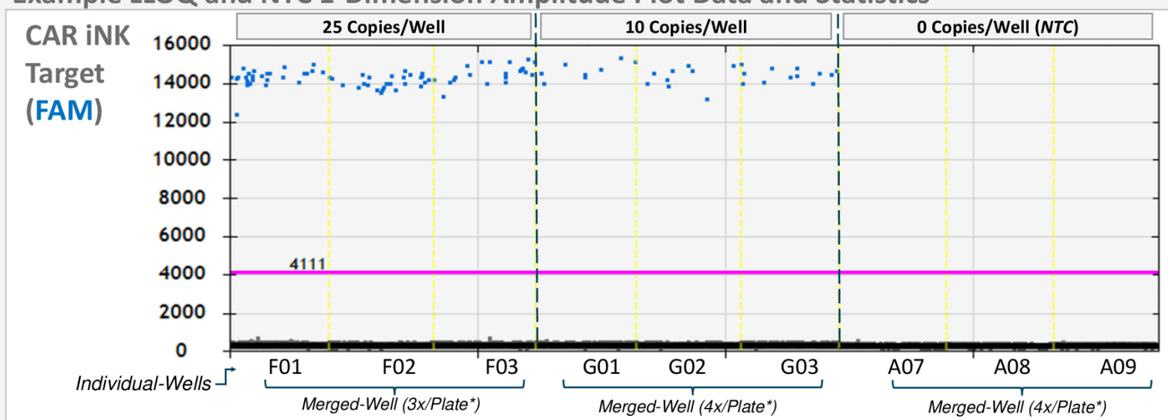
The lowest concentration standard where quantification was accurate, precise, and linear when using either individual-well or merged-well analysis was validated as the Lower Limit of Quantification (*LLOQ*). The Limit of Blank (LOB) and Limit of Detection (LOD) were calculated statistically via conventional formulae[(1)].

Using conventional individual-well analysis, the CAR iNK target assay was validated with a LLOQ & LOD of 25 & 12 copies/well respectively. Merged-well analysis reduced the CAR iNK target LLOQ by 60%, and the CAR iNK Target LOD by 56%. Using merged-well analysis, the CAR iNK target assay was validated with a LLOQ and LOD of 10 and 4.4 copies/well.



Merged-well analysis achieved a 60% reduction in the CAR iNK target LLOQ and a 56% reduction in the CA iNK target LOD.

Example LLOQ and NTC 1-Dimension Amplitude Plot Data and Statistics



25 Copies/Well	CV%	RE%
Individual-Well	23.4%	0.4%
Merged-Well	17.0%	-0.3%

10 Copies/Well	CV%	RE%
Individual-Well	42.3%	0.9%
Merged-Well	20.4%	-0.7%

**: Reported statistics are for a whole plate. Replication of the tested samples varied in the performed analysis. 25 copies/well was evaluated in 9x Individual- and 3x Merged-Wells per plate. 10 Copies/Well and the NTC were each evaluated in 12x Individual- and 4x Merged-Wells per plate.*

CONCLUSION(S)

While both individual-well and merged-well analysis resulted in sufficient assay performance for PK monitoring of CAR iNK, merged-well analysis achieved approximately 60% reduction in both the LLOQ and LOD of the CAR iNK assay.

Improved CAR iNK target assay sensitivity via merged-well analysis was primarily traceable to a reduction in the CV% determined for low concentration standards (*e.g. 10 copies/well*). This improved precision is due to increased overall droplet count, allowing for more accurate calculation of copy number when positive droplets are rare. Despite merged-well analysis having reduced number of replicates ($n = 4$) compared to that of single-well analysis ($n = 12$), each value derived from the merging of three wells is more precise.

Evaluation of CAR iNK cellular kinetics via conventional, triplicate, ddPCR analysis using routine sample acceptance criteria and individual-well analysis could fail to produce valid results in patient PBMC samples where either the total cell number is low (*shortly after lymphodepletion*) or the CAR iNK abundance is low relative to the native PBMC population (*after recovery from lymphodepletion*). In many such cases, evaluation of the obtained data via merged-well analysis would be expected to yield valid quantification or detection data, which would permit improved monitoring of both initial CAR iNK cell expansion and persistence.

While there are regulatory guidelines for bioanalytical method validation [(2,3)], concrete recommendations for the validation of ddPCR assays are lacking. We demonstrate that the methodology presented here is suitable for the analysis of cellular kinetics.

REFERENCE(S)

1) Armbruster DA, Pry T. Limit of blank, limit of detection and limit of quantitation. Clin Biochem Rev. 2008 Aug;29 Suppl 1(Suppl 1):S49-52.

LOB = Mean NTC$_{(Copies/well)}$ + 1.645 × NTC Standard Deviation

LOD = LOB$_{(Copies/well)}$ + 1.645 × LLOQ Standard Deviation

2) US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research. (2018) "Guidance for Industry: Bioanalytical Method Validation." US FDA, Rockville, MD.

3) European Medicines Agency, Committee for Medicinal Products for Human Use, 192217 (2011) "Guideline on Bioanalytical Method Validation." London, UK.

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