Development and Qualification of Multiplexed ddPCR Assay to Evaluate DNA Integrity

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Introduction

Droplet digital PCR (ddPCR) has become a standard method to obtain titer of gene therapy products to determine dose. However, traditional ddPCR only targets a small ~100 base pair length of the genome, giving us little information on the length or intactness of the entire genome. Multiplex ddPCR has been developed as an analytical method to determine how much gene product is intact and if any product is potentially fragmented by using two or more primer sets targeting areas spanning the genome from end to end. Determining the % Intact of a drug product can lead to better understanding of products potency, stability and efficacy.

Methods and Materials

To measure the intactness of a drug product population, a multiplex ddPCR method was developed. Multiplex ddPCR is similar to traditional ddPCR, with the addition of a second primer set with a different fluorescence tag. To utilize this method for measuring % Intact genome in a drug, the primer sets were designed to target the 5' and 3' ends of the genome. When analyzing the results, if there is amplification for both primer sets, it is considered a whole gene and is deemed intact. ddPCR can separate the reaction into about 20,000 oil droplets before amplification, allowing visualization of 1 genome per droplet. To accurately capture % Intact genome correctly, digestion with restriction enzymes is necessary. To qualify this method, the parameters tested include linearity, accuracy, intermediate precision and repeatability. In addition, primer sets across the packaged DNA indicate that strand breaks occur evenly across the gene of interest and not at a specific "hot spot".

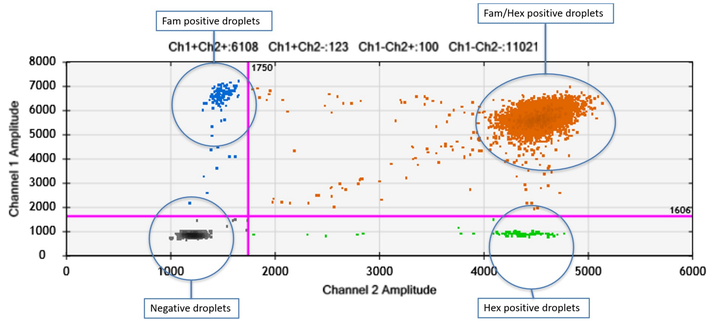

Figure 1. 2D Amplitude graph of positive droplets for a Multiplex ddPCR run. Positive droplets in both channels indicates presence of both primer sets, a that whole gene is considered intact.

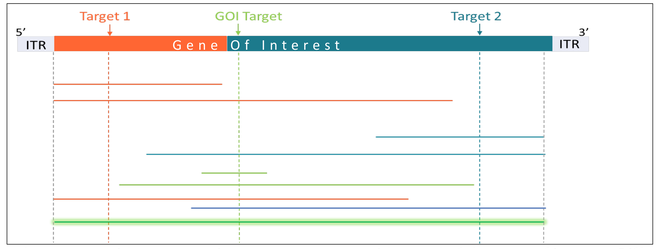

Figure 2. An example genome with our two primer targets, one on either end of the gene of interest. Different line lengths represent theoretical examples of partial fragments of the genome, and the bottom green line represents a complete intact genome.

Linearity for Heat Degradation Samples

Assay linearity was assessed using samples incubated at 25°C to promote genome degradation. The longer the samples were held at 25°C, the lower the % Intact genome.

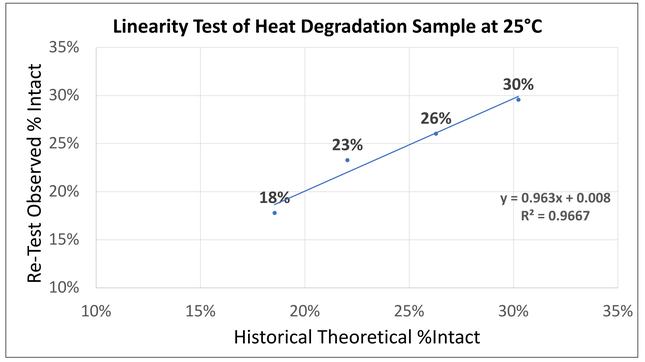

Figure 3. Linearity of historical and observed % Intact data of heat-degradation samples held at 25°C from 14 days to 90 days. Each data point represents the % Intact at a different time point from 14, 30, 60, and 90 days respectively, from right to left. % Intact decreases as samples were heat treated for longer periods of time.

Accuracy and Intermediate Precision

Assay accuracy experiments were run to assess precision and repeatability, as well as the intraassay variability of multiplex ddPCR. A single sample was plated in seven replicates and run through the accuracy assay using 3' and 5' end primer sets. The accuracy assay was then repeated by another analyst on a different day to generate intermediate precision and repeatability results.

	3' Titer (vg/mL)	5' Titer (vg/mL)	% Intact
Average	2.01E+10	2.55E+10	43%
%CV	5%	4%	3%

Table 1. Multiplex ddPCR results for accuracy assay and intermediate precision are summarized above with the average 3' and 5' titers and % Intact for all 13 replicates across two plates. The results show the intraassay variability for this assay is 3% and that the assay is precise and repeatable.

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Strand Breaks Across Gene of Interest

To assess if there were any "hot spots", primer sets were designed to "walk" along the genome, each one separated by about 200-400 base pairs. Adjacent primer sets were run on the multiplex method to determine if any regions of the genome had significantly lower intact genome.

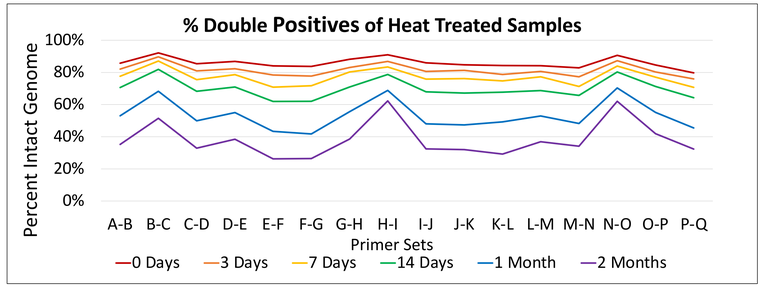

Figure 4. Multiplex ddPCR data of heat-treated drug substance samples held at 40°C from 0 days to 2 months. % Intact decreased as samples were held at 40°C for longer periods of time. The different primer sets that span the gene of interest showed no significant difference in the % Intact.

Multiplex Compared to Expression Assay

To assess whether % Intact genome correlates to therapeutic protein expression, multiplex ddPCR and a cell-based expression assay were compared using long term frozen samples. If protein expression dropped over time, % Intact genome was expected to drop as well.

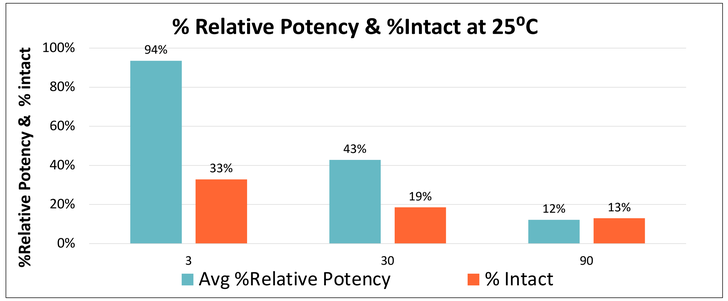

Figure 5. Multiplex ddPCR results of % Intact compared to Average % Relative potency (protein expression) results for stability samples held at 25°C from 3 days to 90 days. Both % Intact and Average % Relative potency dropped as samples were held at 25°C for longer periods of time, indicating multiplex ddPCR generated % Intact correlated with cell-based protein expression.

Conclusion

Through development and pre-qualification of Multiplex ddPCR, we have shown that the method quantified the intact gene of interest, and that % Intact correlated with cell-based protein expression. Precision and repeatability have been demonstrated through the development and pre-qualification experiments for use of DNA characterization in product characterization and stability indications. Hot spot analysis did not identify any regions of concern suggesting that any genome fragmentation occurring is random.