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Quantification of residual BHK DNA by a novel droplet digital PCR technology

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ABSTRACT

For drug substances manufactured in cell lines, host cell DNA is a common contaminant and its level must be carefully monitored. While residual DNA assays have been developed for many production cell lines, a robust assay is unavailable for baby hamster kidney (BHK) cells. The lack of genomics data of Syrian hamster, the origin of BHK cells, makes it challenging to design primers and probes for PCR-based methods. In this paper, we identified intracisternal A-particle (IAP) as an efficient PCR target for BHK DNA. PCR against IAP has been tested with conventional qPCR as well as with the recently developed ddPCR method, both of which demonstrated good efficiency with purified BHK DNA. However, the ddPCR-based method is less prone to matrix interference and is significantly more accurate than qPCR when testing complex samples, including multiple process intermediates. This study not only established a robust assay for the detection of residual BHK DNA, but also evaluated the capability of ddPCR technology for a new application.

1. Introduction

Many biopharmaceutical products, including recombinant protein, vaccines and gene therapy vectors, are derived from expression systems involving host cells. Residual host cell DNA is a major safety concern due to its tumorigenic or infectious potential [1,2] and must be reduced to an acceptable level during downstream purification. The World Health Organization (WHO) guidelines allow residual host cell DNA of no more than 10 ng per dose [3], and similar requirements are enforced by the US Food and Drug Administration (FDA) and European Union (EU) [4,5]. To meet these requirements, highly sensitive methods are needed to quantify low level of host DNA. So far, qPCR targeting repetitive DNA sequences (i.e. Alu repeats) is the standard approach in the industry.

Baby hamster kidney (BHK) is a fibroblast cell line derived from the kidneys of 1-day-old Syrian golden hamsters. Because it is permissive to transfection and viral infection, genetically stable and adaptable to scale-up culture in bioreactors, BHK is one of the most widely used cell lines for the expression of biopharmaceuticals [6,7]. Chinese hamster ovary (CHO) is another popular cell line in the biopharma industry, and is derived from a different species of hamster. Residual DNA assays for CHO cells have been developed and have shown great sensitivity [8,9]. In contrast, there is only one published method on residual BHK DNA and details of assay performance are not available [10]. Because of the lack of genomics data for the Syrian golden hamster, it is not clear how conserved it is with Chinese hamster, or whether primers and probes designed for CHO cells can be applied to BHK cells. It also poses significant challenges for designing primers and probes specific for BHK.

Residual DNA assays typically use qPCR, but a recent attempt with the new Droplet Digital PCR (ddPCR) technology produced encouraging results [11]. While qPCR reads the total signal, ddPCR partitions a PCR reaction into 20,000 nanoliter-sized droplets, and therefore is more resistant to PCR inhibition and bias [12,13]. After PCR amplification, each droplet is analyzed in a FACS-like manner and the final result is derived from tens of thousands of data points, which ensures great precision [14,15]. In this study, we identified intracisternal A-particle (IAP) as an efficient and specific PCR target for BHK DNA. IAP is a class of transposable elements with about 1000 copies in the rodent genome [16]. The performance of IAP qPCR and ddPCR have been evaluated by testing both purified and complex samples, and ddPCR was found to be more advantageous for the development of a residual BHK DNA assay.

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2. Experimental procedures

2.1. BHK DNA standard

Genomic DNA of BHK cells was extracted with the DNeasy Blood & Tissue kit (Qiagen) with additional purification by phenol-chloroform extraction. DNA concentration was determined by Picogreen dsDNA kit (Molecular Probes) and DNA was stored at −70 °C.

2.2. Primers and probes

Sequences of PCR primers and probes for Alu [17] and 5S rRNA [10] were previously published. IAP primers and probe were designed by PrimerQuest (Integrated DNA Technologies). All oligos were ordered from ThermoFisher Scientific.

CHO Alu Primer F	5'- CTACCAGAGGTCCTGAGTTCAATTC -3'
CHO Alu Primer R	5'- GGGCACCAGGTCTCATAACG -3'
CHO Alu Probe	5'-(FAM)-CAGCAACTACATGGTGGCTCACAACCA-(TAMRA)-3'
5S rRNA Primer F	5'-CGCAGCAGCAGGCTCT-3'
5S rRNA Primer R	5'-ACCCTGCTTAGCTTCCGAGA-3'
5S rRNA Probe	5'-(FAM)-CCGCCGTCGTCTACGGCCATACC-(TAMRA)-3'
IAP Primer F	5'-TTTGCCTTCACTATCCCTTCTC-3'
IAP Primer R	5'-GCTGACATATTGTTGGGCTATTG-3'
IAP Probe	5'-(FAM)-ATTTCAGTGGAAGGTACTGCCGCA-(TAMRA)-3'

For ddPCR, MGB was used as the quencher instead of TAMRA to minimize fluorescent background.

2.3. qPCR and data analysis

qPCR reactions were prepared with TaqMan Universal Master-Mix in a 96-well optical plate (Life Technologies). The 50 μL PCR mixture contains final concentrations of primers and probe at 1 μM and 0.25 μM, respectively. DNA standard and test samples were diluted in ddH2O to required concentration or dilution factor. qPCR program: 1 cycle of: 50 °C × 2′, 95 °C × 15′, 40 cycles of: 95 °C × 30″, 60 °C × 1′ was conducted in ViiA™ 7 Real-Time System (Life Technologies). qPCR data were automatically analyzed by the ViiA 7 software. Ct values and calculated DNA concentrations were reported.

2.4. ddPCR and data analysis

ddPCR reactions were prepared with ddPCR Supermix for Probes (No dUTP) following the instructions from Bio-Rad. DNA standard and test samples were diluted in ddH2O to required concentrations or dilutions. Final concentrations of primers and probe were 0.9 μM and 0.25 μM, respectively. After droplet generation, the following PCR program was run: 1 cycle of 95 °C × 10′; 40 cycles of 94 °C × 30″, 60 °C × 1′, and 1 cycle of: 98 °C × 10′; 4 °C hold. PCR results were analyzed by QX200 Droplet reader and QuantaSoft (Bio-Rad).

3. Results

3.1. Identification of a PCR target for BHK DNA

After extensive study of literature, only one report was found to describe the method of detecting residual BHK DNA, which is based on qPCR against 5S rRNA [10]. The published method was tested but the assay performed poorly in our hands (Data not shown). qPCR against the repetitive Alu sequence has been used to detect residual CHO DNA, and the Alu primer/probe set might be applied for BHK DNA depending on the sequence homology between Chinese and Syrian hamsters. A third option is to design primers and probes specific for BHK DNA. Although the genome information for BHK cells is very limited, the DNA sequence of a transposable element named intracisternal A-particle (IAP) was reported [18]. In order to identify the best PCR target, primers/probe sets against the sequences mentioned above were put to a head-to-head comparison. As shown in Fig. 1A, when using CHO DNA as template, qPCR amplification of Alu was very efficient, followed by 5S rRNA, while IAP failed to amplify at all. In contrast, when using BHK DNA as template, IAP qPCR was significantly more efficient than 5S rRNA and Alu (Fig. 1B). IAP primers and probe were further evaluated with DNA from other cell lines used in drug manufacturing, including HeLa, HEK293 and Vero. No PCR amplification was observed with DNA from cells other than BHK (Fig. 1C). These results demonstrate not only the efficiency, but also the specificity of IAP PCR for BHK DNA.

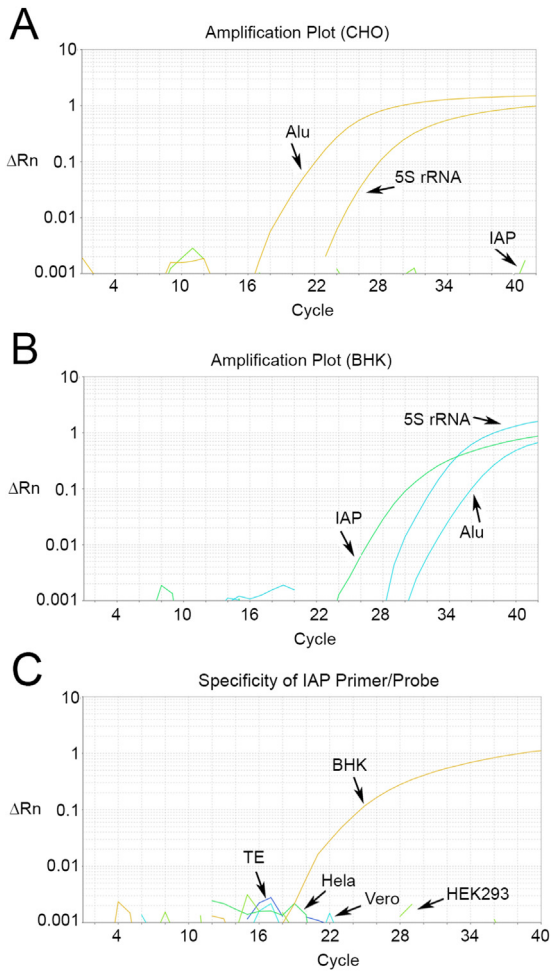

Fig. 1. Efficiency and specificity of IAP primer/probe set for BHK DNA.
(A) Primers and probes designed for BHK 5S rRNA and IAP, as well as CHO Alu, were tested on the qPCR platform using CHO DNA as template. Plot demonstrated efficient PCR amplification against Alu and no amplification for IAP.
(B) Same tests as in (A) were performed using BHK DNA as template. PCR amplification against IAP is more efficient that 5S rRNA and Alu.
(C) IAP PCRs were conducted against DNA from BHK, HeLa, HEK293 and Vero cells, or TE buffer as negative control.

3.2. Detection of BHK DNA by qPCR

BHK DNA was first analyzed with the conventional qPCR method. BHK DNA standard was prepared in ddH2O to a concentration of 1E+4 pg/mL, which was further 1:10 serially diluted in ddH2O until 1 pg/mL. 25 μL samples were analyzed in 50 μL qPCR reactions. As shown in Fig. 2A, good dilutional linearity was observed in all samples analyzed, with a regression coefficient of 0.9967. The dynamic range spanned across 4 orders of magnitude