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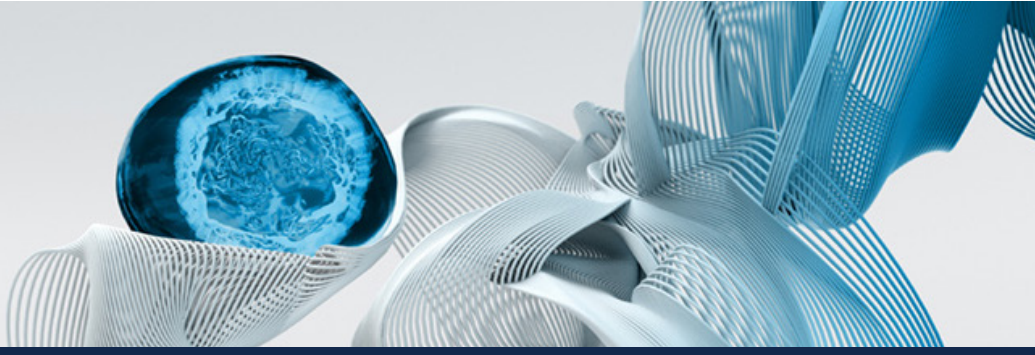

Technical Note

Real-time PTA

Cells explored. Answers revealed.

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Optimization of whole genome amplification parameters with real-time Primary Template-directed Amplification (rtPTA).

Primary Template-directed Amplification (PTA) is a novel and accurate whole genome amplification (WGA) method for the genomic analysis of single cells and ultra-low input DNA samples. The optimal amplification time for specific sample types, applications or laboratory settings may be determined empirically by performing the reaction in a real-time format.

Introduction

Despite significant advances in genomics analysis methods, hundreds of nanograms to microgram quantities of DNA are still needed for many NGS sample preparation workflows. When working with single cells or other limited-input samples, picogram quantities of genomic DNA have to be amplified to provide the desired amount of input material.

Primary Template-directed Amplification (PTA) is a novel, isothermal method that overcomes the challenges associated with WGA, such as low and/or uneven genome coverage, allelic skewing or dropout, and experimental artifacts.[1]

The standard PTA protocol[2] calls for an amplification time of 10 hours. In certain applications, shorter or longer amplification times may be needed to optimize the yield from the PTA reaction for downstream processing, or may be desired to accommodate specific laboratory workflows. More importantly, the ploidy of different cell types may impact the course and outcome of the amplification reaction.

In this Note, we provide reaction conditions for performing PTA in a real-time format (Figure 1), as well as guidelines for the empirical optimization of amplification time. This approach is highly recommended during method development and validation, and prior to processing large numbers of precious samples.

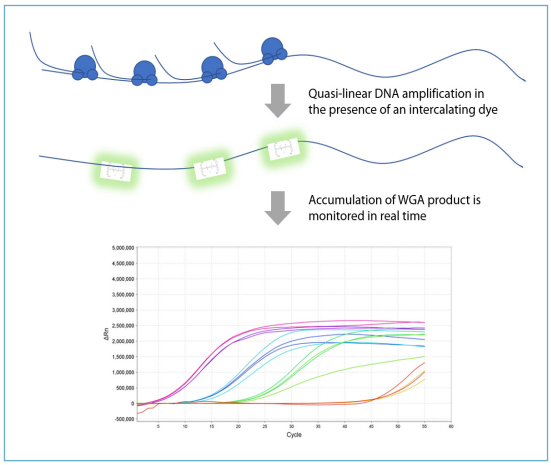

Figure 1. The principle of Real-time PTA. WGA is performed in the presence of an intercalating dye. Fluorescence is monitored in real-time to determine the optimal amplification time for a specific input, sample type or application.

1 Gonzalez V, et al. bioRxiv 2020.11.20.391961; doi: 10.1101/2020.11.20.391961
2 ResolveDNA™ Whole Genome Amplificaton Kit Protocol P00001

For Research Use Only. Not for use in diagnostic procedures.

Materials and Methods

To demonstrate the principle and utility of real-time PTA (rtPTA), different amounts of human genomic DNA or sorted cells were amplified with the Resolve DNA™ Whole Genome Amplification Kit (BioSkryb Genomics, cat. no. 100068 or 100136).

Single human adenocarcinoma SKBR3 cells (ATCC® HTB-30™) were "dry-sorted" with an SH800S Cell Sorter (Sony Biotechnologies), into column 6 (1 cell per well), column 7 (3 cells per well) and column 8 (5 cells per well) of a Low Bind 96-well PCR plate (BioSkryb Genomics, cat. no. 100149). Eight replicates of each condition were prepared, as indicated in Figure 2.

Human genomic DNA (50 ng/µl; included in the ResolveDNA WGA kit) was diluted to 1 ng/µl, 100 pg/µl or 10 pg/µl in ResolveDNA PTA-Grade Elution Buffer (BioSkryb Genomics, cat. no. 100178). Of each dilution, 1 µl was dispensed in quadruplicate, according to the plate layout in Figure 2.

rtPTA reactions were assembled as outlined in Table 1. The only modification from the standard PTA protocol is the inclusion of EvaGreen™ Dye (Biotium, cat. no. 31000-T), which enables real-time monitoring of amplification. Quadruplicate no-template control (NTC) reactions and a single no-dye NTC (noEG) were included in the reaction scheme.

Real-time PCR was performed using a QuantStudio™ 7 Flex Real-Time PCR System (ThermoFisher Scientific), with the following cycling parameters:

- Lid temperature: 70°C
- 56 cycles of 30°C for 15 min (14 hours total)
- Hold at 4°C
- Data collection (SYBR Green/FAM channel): every 15 min

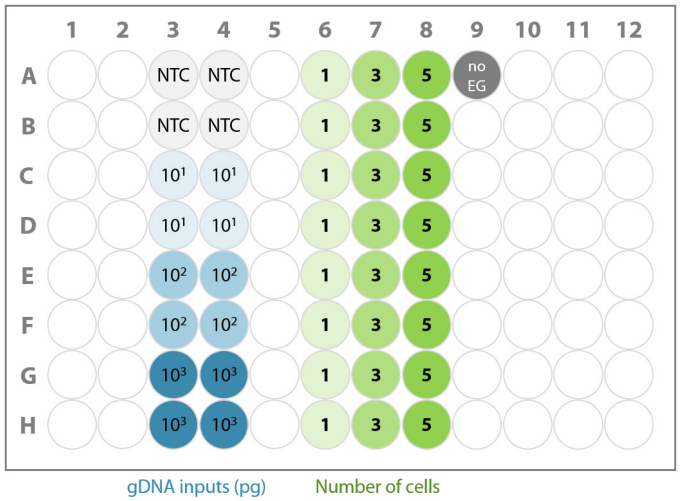

Figure 2. Plate layout. Cells (1, 3 or 5; n = 8) were sorted into empty (dry) wells of columns 6 to 8, whereas diluted gDNA (10 pg, 100 pg or 1 ng; n = 4), was dispensed into individual wells of columns 3 and 4. NTC = no template control (n = 4) and no EG = NTC without EvaGreen (n = 1).

The plate was used immediately, but dry-sorted cells may be frozen on dry ice and stored at -80°C if the rtPTA reaction can only be set up at a later stage.

Table 1. Real-time PTA reaction setup.

Component/ volume	Single cells	gDNA controls	NTCs	noEG NTC
Diluted human gDNA[a]	N/A	1.0 µL	N/A	N/A
Single or multiple cells (dry sorted)	1, 3, or 5	N/A	N/A	N/A
ResolveDNA PTA-Grade Cell Buffer[b]	2.5 µL	1.5 µL	2.5 µL	3.0 µL
EvaGreen Dye[c]	0.5 µL	0.5 µL	0.5 µL	N/A
Total volume	**3.0 µL**	**3.0 µL**	**3.0 µL**	**3.0 µL**

a Amount of input DNA per well was 10 pg, 100 pg or 1 ng as per the plate layout in Figure 2.
b **Do not** pipette up and down to mix well contents after Cell Buffer is added to cells.
c **Do not** exceed the recommended volume of EvaGreen.

Results

As shown in Figure 3, the isothermal PTA reaction can be monitored in real time through the incorporation of an intercalating fluorescent dye. The amplification time required to obtain a detectable signal is proportional to the amount of input DNA or number of cells, and varied between <1 h and 5 h for the gDNA inputs (1 ng – 10 pg) used in this study. SKBR3 cells are hypertriploid and produced a detectable signal between 2 h and 4 h of amplification, depending on the number of cells used. The time needed to pass the detection threshold is expected to be longer for diploid cells.

It is important to note that NTC signals remained undetectable until 10 h of amplification, but reached a significant level after 14 h.

When the amplification curve passes the detection threshold, the yield of amplified DNA is estimated to be approximately 100 ng. Once the rtPTA signal reaches a plateau, low microgram quantities of DNA have been produced (data not shown). This suggests that a sufficient amount of material for library preparation is available shortly after a signal is

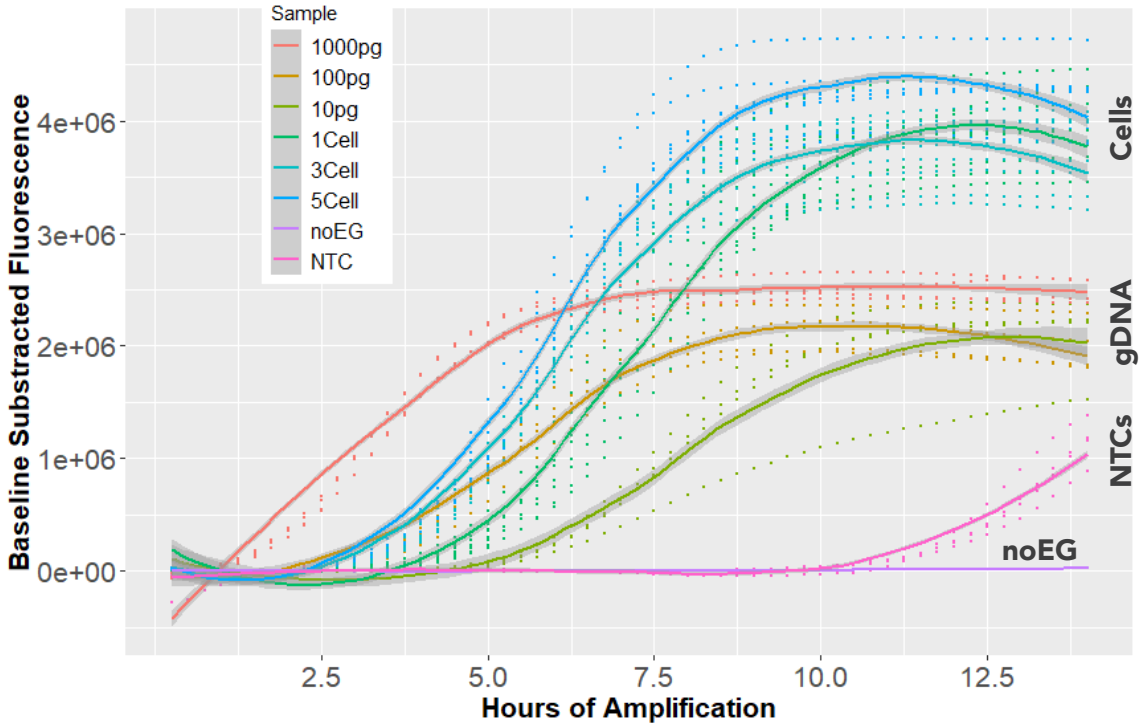

Figure 3. Real-time PTA curves for SKBR3 cells, genomic DNA and no-template controls. All inputs produced a detectable signal between <1 h and 5 h of amplification, whereas no template control (NTC) reactions only crossed the threshold after 10 h. NoEG = No EvaGreen NTC.

detected. The optimal PTA amplification time for a specific amount of input DNA, sample or cell type should therefore be selected to:

- fall within the linear phase of the reaction (after a signal is detected, but before the plateau is reached)
- be shorter than the time needed to produce a detectable signal from NTC reactions

The data generated in this study suggest that it may be possible to reduce the standard 10-h PTA time significantly when working with high-picogram inputs of purified DNA. For sorted cells, optimal amplification times are likely to fall within the 5 – 10 h range. If different types of inputs are required to satisfy experimental design, select a time that meets all of the above criteria for the entire set of inputs to enable streamlined processing of samples.

Excessive amplification beyond 10 hours should be used with caution to limit the potential formation of artifacts that may impact sequencing data quality and downstream analysis.

Conclusions

Real-time PTA is a simple method for the optimization of complex WGA conditions that can be performed with standard molecular biology laboratory equipment. The method enables real-time monitoring of the accumulation of both bona fide reaction product and undesired, non-specific amplification products. The optimal amplification time for a project will be determined by the type and range of inputs, and may be tailored to ensure efficiency within operational constraints.

Real-time PTA is also recommended for:

- proof-of-concept testing prior to adopting PTA in different applications or laboratory workflows
- the detection of microbial or human genomic DNA contamination in molecular diagnostics laboratories
- monitoring of bioprocessing contaminants during protein or vector production

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