Monitoring of tisagenlecleucel transgene DNA using a quantitative polymerase chain reaction assay

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Chimeric antigen receptor (CAR)-T cell therapies reprogram T cells to engage and eliminate cancer cells. Patients’ T cells are transduced in vitro using lentiviral or retroviral vectors containing a CAR transgene. Following infusion, CAR-T cells expand in vivo and may persist in the peripheral blood and bone marrow for years. Therefore, monitoring in vivo copies of the CAR transgene requires highly sensitive, validated analytical methods. Herein, we describe the validation of a qPCR assay to detect tisagenlecleucel transgene in patient samples. The limit of detection and lower limit of quantitation were 3.1 and 10 copies/200 ng genomic DNA, respectively, equivalent to ~50 copies/μg genomic DNA and in alignment with US Food and Drug Administration guidance on bioanalytical method validation. The assay allowed quantitation of the tisagenlecleucel transgene over a wide dynamic range with a high degree of linearity, that is, 101–106 copies/200 ng genomic DNA (R2 ≥ 0.9988). Coefficients of variation of measured transgene copies ranged from 0.2% to 12.8%. A droplet digital PCR assay was performed as a method of validation and showed a strong correlation with the qPCR assay (R2 = 0.9980, p < 0.0001). This qPCR assay is being utilized to monitor tisagenlecleucel expansion and persistence in clinical trials.

INTRODUCTION

Chimeric antigen receptor (CAR)-T cell therapies are a new class of immunocellular therapies that genetically engineer T cells to engage and eliminate cancer cells. Tisagenlecleucel is an autologous CD19-directed CAR-T cell therapy that is approved for the treatment of pediatric and young adult patients with relapsed or refractory (r/r) B cell acute lymphoblastic leukemia (ALL) and adult patients with r/r diffuse large B cell lymphoma (DLBCL).1–4 The manufacturing process for CAR-T cells begins with apheresis, a technique in which leukocytes are isolated from a patient’s blood. T cells are then enriched and transduced with genetically engineered lentiviral or retroviral vectors in vitro. The sequence encoding the CAR transgene contains an antibody fragment for antigen binding, an extracellular hinge, a transmembrane domain, and co-stimulatory and intracellular T cell signaling domains. The expressed CAR transgene promotes the activation and expansion of the CAR-T cells, and it allows them to engage and eliminate cells that express the target antigen. The CAR-T cells are expanded in vitro and ultimately infused back into the patient.5–7

CAR-T cells expand multiple logs beyond the administered cell dose and may persist in the peripheral blood for years following infusion.8,9 Therefore, it is critical to have quantitative bioanalytical methods to accurately detect and monitor the in vivo cellular kinetics of CAR-T cells with high sensitivity. Quantitative polymerase chain reaction (qPCR) offers an ideal methodology for monitoring CAR transgene expression that can accurately measure the number of CAR transgene copies, provide high sensitivity and specificity over a wide dynamic range, and provide compatibility with a range of different sample types.10

This report describes the validation of a modified qPCR assay, originally described by Kalos et al.,6 designed to specifically identify the tisagenlecleucel CAR transgene in peripheral blood, bone marrow, cerebrospinal fluid (CSF), lymphatic tissue, and other tissue types. In addition, a cyclin-dependent kinase inhibitor 1A (CDKN1A) copy number qPCR assay was developed and validated as a reference gene assay to correct for variability in the samples’ DNA input and quality. Both qPCR assays were validated according to industry guidelines, including the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines.11 A droplet digital PCR (ddPCR) version of the qPCR assay was also developed to allow for absolute quantitation of tisagenlecleucel transgene DNA without the need of a standard curve. The ddPCR assay was used to assess and validate the accuracy of the qPCR assay as well as to enable accurate value assignment of the qPCR calibrators and controls.

RESULTS

qPCR assay limits

The limit of blank, limit of detection, and lower limit of quantitation of the qPCR assay were evaluated by testing a dilution series of the...
ddPCR value-assigned calibrator DNA (0, 0.6, 1.3, 2.5, 5, 10, 25, and 50 transgene copies/200 ng human genomic DNA [gDNA]). The serial dilutions were prepared three times, and nine replicates of each concentration were tested for a total of 27 replicates in three consecutive qPCR runs. In addition, triplicates of each of the eight calibrators (0, 10, 50, 100, 1,000, 10,000, 100,000, and 1,000,000 copies/200 ng human gDNA) were included in each qPCR run. The resulting standard curve was used to quantitate the copy numbers of each of the tested serial dilutions, and to calculate recovery rates and precision of the qPCR assay.

None of the 27 replicates containing 0 transgene copies/200 ng human gDNA showed a signal above the Ct cutoff after 40 amplification cycles. Hence, the limit of blank was determined to be 0 transgene copies/200 ng human gDNA. The limit of detection of the qPCR assay was estimated to be 3.1 transgene copies/200 ng human gDNA, representing the lowest concentration at which transgene DNA can be measured with a 95% detection rate (Figure 1). The minimum concentration or lower limit of quantitation at which transgene DNA can be quantitated with an acceptable recovery rate (80%–120%), precision (coefficient of variation [CV] <35%), and a 100% detection rate was determined to be 10 transgene copies/200 ng human gDNA (Table 1). This equates to approximately 50 transgene copies/μg gDNA, which is consistent with US Food and Drug Administration (FDA) guidance on bioanalytical method validation.12

**Accuracy**

The accuracy of the qPCR assay was assessed using 47 specimens, including spiked blood or bone marrow samples containing different amounts of CAR-transformed Jurkat cells, CAR plasmid DNA, and CAR-transformed T cells from normal blood donors or patient samples. qPCR results were compared with results from a ddPCR assay, which used the same primer and probe sequences. The resulting R² value (0.9980 with p < 0.0001) indicated a strong correlation between the qPCR and ddPCR assay results (Figure 2).

**Linearity and dynamic range**

The linearity and dynamic range of the tisagenlecleucel transgene qPCR assay were evaluated and compared with a qPCR assay using CDKN1A primers and probe as an internal control. Both CDKN1A and tisagenlecleucel qPCR assays were run in parallel on the same qPCR plates. Representative standard curves for both the tisagenlecleucel transgene and CDKN1A qPCR assays are shown in Figure 3. The mean R² value (0.9988 ± 0.0008) from three independent tisagenlecleucel transgene qPCR assay runs indicated a high degree of linearity, defining a dynamic range of 10–1,000,000 transgene DNA copies/200 ng human gDNA. The mean slope of –3.3870 ± 0.0259 resulted in a mean amplification efficiency of 97.36% ± 1.02%. Similarly, the observed mean R² value (0.9980 ± 0.0011) from three independent CDKN1A qPCR assay runs demonstrated good linearity and acceptable mean slope (–3.3138 ± 0.0676) and mean amplification efficiency (100.39% ± 2.89%).

**Variability**

We assessed the intra-assay and inter-assay variability of the qPCR assay by testing eight blood and bone marrow specimens from normal donors spiked with different amounts of CAR-T plasmid DNA. Variability was also assessed using CSF and formalin-fixed, paraffin-embedded (FFPE) tissue samples. Three independent runs were performed, and each sample was run in triplicate. The CV for intra-assay variability ranged from 0.2% to 7.2%, and the CV for inter-assay variability ranged from 1.6% to 12.8% (Table 2).

**DISCUSSION**

Highly specific, sensitive, and quantitative analytical assays are needed to monitor the in vivo cellular kinetics of CAR-T cells, including their expansion and persistence in the patient after infusion. It is important that these assays are also amenable to a variety of different sample types, such as whole blood, bone marrow, CSF, and other tissue types. In addition, the ability to use common shipping methods for patient samples, short turnaround time, and high-throughput capability are especially advantageous in a
Table 1. Lower limit of quantitation of the tisagenlecleucel transgene qPCR assay

| Input copy number | 50.0 | 25.0 | 10.0* | 5.0 | 2.5 | 1.3 | 0.6 | 0  |
|-------------------|------|------|-------|-----|-----|-----|-----|----|
| Detection rate    | 100.0% | 100.0% | 100.0% | 100.0% | 92.6% | 70.4% | 37.0% | 0.0% |
| Measured copy number mean | 48.5 | 25.4 | 9.8  | 5.1 | 3.1 | 2.4 | 1.3 | N/A |
| Quantity transgene copy number CV | 14.7% | 20.3% | 31.8% | 39.9% | 54.3% | 55.4% | 39.1% | N/A |
| Recovery transgene copy number | 97.0% | 101.4% | 97.8% | 102.3% | 122.8% | 190.4% | 203.7% | N/A |

Measured detection rates, transgene copy number means, CVs, and recovery rates for 0, 0.6, 1.3, 2.5, 5, 10, 25, and 50 copies of transgene DNA/200 ng human gDNA are shown. CV, coefficient of variation; gDNA, genomic DNA; qPCR, quantitative polymerase chain reaction.

*Entries in this column show the determined lower limit of quantification.

manufacturing environment and during posttreatment follow-up. Therefore, a qPCR-based assay provides an ideal solution for monitoring transgene presence.

We developed a qPCR assay for the detection of tisagenlecleucel transgene DNA that is highly sensitive, reproducible, and linear over five orders of magnitude. We also demonstrated high specificity of the assay and a strong correlation between the qPCR and calibrator-independent ddPCR assay results. DNA isolated from whole blood, bone marrow, and transfected T cells was successfully tested in this study. Other sample types (such as frozen tissue [data not shown], FFPE tissue, and CSF) were also successfully quantified using the qPCR assay.

The reference CDKN1A qPCR assay was run in parallel and successfully used to detect and adjust for potential DNA quantity errors and quality challenges using 5–10 ng of input gDNA per reaction. In addition, a correction factor based on the measured input and output gDNA concentration was used to adjust the transgene copy number. This allowed for the accurate calculation of copies of transgene per tested gDNA amount, and for the correct estimation of transgene copies per volume of whole blood and bone marrow. Another critical factor in obtaining the most accurate test results was value assignment of the DNA calibrators used in both tisagenlecleucel transgene and CDKN1A qPCR assays by ddPCR. Taken together, these data demonstrate that the qPCR assay is sensitive, accurate, and can detect tisagenlecleucel transgene DNA in a variety of biological sample types.

The qPCR assay described herein has been used in multiple clinical trials to evaluate the cellular kinetics of tisagenlecleucel and has clearly demonstrated the utility of this method.1,2,3,9,13 The qPCR-based approach was used to evaluate the cellular kinetics of tisagenlecleucel in pediatric and young adult patients with B cell ALL to better understand the significance of CAR-T cell expansion and persistence in relationship to response.1 In that study, transgene DNA copies were found to be approximately 2-fold higher in patients who responded to tisagenlecleucel compared to nonresponders, suggesting an association between response and higher tisagenlecleucel expansion. Furthermore, tisagenlecleucel transgene DNA was detectable beyond 2 years in responding patients, confirming the high sensitivity of the qPCR assay in clinical samples.

In a separate report of patients with B cell ALL and chronic lymphocytic leukemia, tisagenlecleucel transgene DNA was detected not only in the peripheral blood and bone marrow, but also in the CSF, which has been suggested to be an important clinical finding considering that no central nervous system relapses have been observed to date in tisagenlecleucel-treated pediatric and young adult patients with B cell ALL.5,14

Different transgene expansion levels have been described between pediatric and young adult B cell ALL and adult DLBCL. A recent study reported lower in vivo expansion of tisagenlecleucel in adult patients with DLBCL compared with that observed in pediatric and young adult patients with B cell ALL, suggesting that there may be differences in CAR-T cell trafficking and/or intrinsic T cell differences between these two patient populations.13 However, higher tisagenlecleucel expansion in adult patients with r/r DLBCL was associated with more severe cytokine release syndrome, and is consistent with what has been observed in pediatric and young adult patients with B cell ALL.8,14 Similar to pediatric and young adult patients with r/r B cell ALL, longer persistence of tisagenlecleucel was observed in adult patients with r/r DLBCL who had a sustained response.3 The utilization of qPCR to track CAR transgene levels in vivo over time will provide additional insight into the relationship between persistence and durability of response to CAR-T cell therapy in different therapeutic settings.

qPCR assays for monitoring of CAR-T cells and detection of replication-competent lentivirus have been recommended by the FDA for cell-based gene therapies.15 In addition to qPCR assays, an array of different CAR-T cell detection methods, such as imaging, enzyme-linked immunosorbent assays, western blotting, and flow cytometry assays, have been used.9,15,16,17 Multiparameter flow cytometry-based approaches provide the benefit of evaluating the form and function (activated versus suppressive) of CAR-T cells as well as the ability to quantify both CAR-T and non-CAR-T cells in the sample. However, in contrast to qPCR, the detection of CAR-T cells by flow cytometry relies on the cell surface expression of the CAR. Hence, transduced T cells without sufficient CAR surface expression might not be identified by flow cytometry, but they could still be detected by qPCR. Indeed, qPCR appeared to be more sensitive than flow cytometry assays in cellular kinetic studies of tisagenlecleucel in peripheral blood, as shorter persistence was detected by flow cytometry.
 compared with qPCR (554 days and 693 days, respectively). Along with lower sensitivity, a reduction in CAR surface expression over time and/or sequestration of circulating CAR-T cells from peripheral blood into tissues may also have contributed to the reduced detection by flow cytometry. We have shown that the combination of high sensitivity, wide dynamic range, accurate quantitation, and compatibility with different sample types provides unique advantages for qPCR assays for the monitoring of transgene DNA in CAR-T cell therapies.

The ability of qPCR-based assays to detect gene expression in a variety of samples has revolutionized their use as molecular diagnostics in the clinic. Our results validate the qPCR-based assay as a viable tool to monitor the cellular kinetics of tisagenlecleucel after infusion. Further evaluation of the tisagenlecleucel transgene in clinical trials and in the real-world setting will improve our understanding of the role of CAR transgene persistence and prolonged response.

MATERIALS AND METHODS

DNA isolation

DNA was isolated and purified from whole blood and bone marrow samples collected in BD Vacutainer K2EDTA tubes (Becton Dickinson, Franklin Lakes, NJ, USA) using QIAamp DNA blood mini kits (QIAGEN, Valencia, CA, USA) for sample volumes below 200 μL and QIAamp DNA blood midi kits (QIAGEN, Valencia, CA, USA) for sample volumes up to 2 mL according to the manufacturers’ protocols. CSF samples were collected in sterile tubes, and DNA was isolated using QIAamp DNA blood midi kits for sample volumes up to 2 mL according to the manufacturer’s protocol. DNA from FFPE tissue samples was isolated using the AllPrep DNA/RNA FFPE kit (QIAGEN, Valencia, CA, USA). Eluted gDNA samples from cytopenic clinical specimens were further concentrated using a MinElute PCR purification kit (QIAGEN, Valencia, CA, USA) according to the manufacturer’s instructions. Isolated gDNA was quantitated using the Qubit double-stranded DNA (dsDNA) broad-range (BR) or high-sensitivity (HS) assay kits (Thermo Fisher Scientific, Carlsbad, CA, USA) and stored at −80°C.

Primer and probe sequences

Published primer and TaqMan non-fluorescent quencher-minor groove binder (NFQ-MGB) probe sequences targeting the junction region of the 4-1BB co-stimulatory domain and the CD3-zeta signaling domain were used in qPCR assays to detect and quantify the transgene in both clinical samples and transduced cell lines. Each assay was validated using a standard curve and a set of calibrators prepared from the same DNA sample. A typical standard curve is shown in Figure 3, which demonstrates the linear relationship between the Log10 transgene copies/200 ng human gDNA and the Log10 ng human gDNA/reaction. The linear fit line is flanked by the 95% confidence interval lines (dotted lines). Each individual calibrator was run in triplicate.

Figure 2. Accuracy of the tisagenlecleucel transgene qPCR assay

Bivariate fit of qPCR data compared to droplet digital PCR (ddPCR) data. The linear fit line is flanked by the 95% confidence interval lines (dotted lines). Copy numbers are normalized to transgene copies/μg human gDNA.

Figure 3. Standard curves of the tisagenlecleucel transgene and CDKN1A qPCR assays

(A and B) Typical standard curves of the (A) transgene and (B) cyclin-dependent kinase inhibitor 1A (CDKN1A) qPCR assays. The linear fit line is flanked by the 95% confidence interval lines (dotted lines). Each individual calibrator was run in triplicate.
region of the tisagenlecleucel transgene, as well as the ubiquitously expressed CDKN1A gene (GenBank: Z85996), are shown in Table 3.5,7 Primers were supplied by Integrated DNA Technologies (Coralville, IA, USA) and probes were supplied by Thermo Fisher Scientific (Carlsbad, CA, USA). Primers and probes were purified by high-performance liquid chromatography according to the manufacturers’ instructions.

DNA standards
Tisagenlecleucel transgene DNA copies in clinical samples were quantitated using an 8-point standard curve (10–1,000,000 lentivirus plasmid copies/200 ng gDNA obtained from healthy volunteer blood donors). Each calibrator of the standard curve was run in triplicate. In addition, a no-template control and a positive control (1,000 lentivirus plasmid DNA copies/200 ng gDNA from healthy volunteer blood donors) were tested in triplicate in each run to assess contamination and quantitation accuracy, respectively. Other run validity criteria included standard curve slopes and $R^2$ values. The lentivirus plasmid DNA calibrator was independently value assigned using ddPCR, which was performed using the same primers and probe as in the qPCR assay.

Every clinical sample was tested in parallel using a CDKN1A qPCR assay to correct for quantitation errors in the initial DNA quantitation by Qubit, but also to control for variability in DNA quality. A 5-point standard curve (0.46–25 ng of gDNA from healthy donors/reaction) was generated in each run, and each calibrator was run in triplicate. The comparison between the expected and observed input amount of gDNA resulted in a correction factor, which was applied to the measured transgene copy number. Results are reported as copies of transgene per input amount of human gDNA and copies of transgene per input volume of either whole blood or bone marrow. Similar run validity criteria were used in the CDKN1A qPCR assay. gDNA calibrators were also value assigned by ddPCR.

**Table 2. Variability of the tisagenlecleucel transgene qPCR assay**

| Sample | Type   | Mean transgene copies/μg human gDNA | CV% | Sample | Type   | Mean transgene copies/μg human gDNA | CV% |
|--------|--------|------------------------------------|-----|--------|--------|------------------------------------|-----|
| A      | WB     | 22,291                             | 1.6 | A      | WB     | 22,474                             | 3.9 |
| B      | WB     | 62,225                             | 0.2 | B      | WB     | 66,439                             | 8.2 |
| C      | WB     | 49,891                             | 1.7 | C      | WB     | 49,027                             | 1.6 |
| D      | WB     | 2,190                              | 7.2 | D      | WB     | 1,928                              | 12.8|
| E      | WB     | 103,541                            | 2.8 | E      | WB     | 110,197                            | 5.5 |
| F      | WB     | 10,266                             | 2.8 | F      | WB     | 10,977                             | 5.8 |
| G      | WB     | 4,119                              | 2.6 | G      | WB     | 4,750                              | 9.8 |
| H      | BM     | 78,785                             | 2.6 | H      | BM     | 81,354                             | 4.9 |
| I      | CSF    | 62,197                             | 7.0 | I      | CSF    | 60,233                             | 3.6 |
| J      | lymphoma FFPET | 19,548                       | 2.0 | J      | lymphoma FFPET | 20,827                       | 6.8 |
| K      | bladder FFPET | 57,795                      | 4.5 | K      | bladder FFPET | 58,111                      | 8.1 |
| L      | kidney FFPET | 31,536                       | 5.9 | L      | kidney FFPET | 33,771                       | 6.9 |

A comparison of intra- and inter-assay variability data was performed. Measured mean transgene copies/μg human gDNA (n = 3) from 12 samples (A–L) and corresponding CV% are shown. BM, bone marrow; CSF, cerebrospinal fluid; FFPET, formalin-fixed, paraffin-embedded tissue; WB, whole blood.

**qPCR protocol**
A TaqMan-based qPCR assay was used to detect tisagenlecleucel transgene DNA in manufactured tisagenlecleucel and clinical samples. Automated qPCR plate set up of the tisagenlecleucel and CDKN1A calibrators/controls, respective qPCR master mixes, as well as clinical samples was performed by a QIAGility instrument (QIAGEN, Valencia, CA, USA). Nine μL of either calibrator/control/clinical sample DNA (200 or 10 ng/reaction for the transgene or CDKN1A qPCR, respectively) or no-template control (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA) was mixed with 10 μL of a 2 x TaqMan gene expression master mix (Thermo Fisher Scientific, Carlsbad, CA, USA) and 1 μL of a transgene or CDKN1A preformulated single 20 x mix containing both forward and reverse primers (900 nM each, final concentration) and probe (250 nM final concentration).

The following amplification/detection conditions were used on the Applied Biosystems ViiA 7 real-time PCR system (Thermo Fisher Scientific, Carlsbad, CA); one cycle at 50°C for 2 min and 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

**ddPCR protocol**
ddPCR reactions were performed using a QX200 AutoDG ddPCR system and C1000 Touch thermal cycler (all instruments from Bio-Rad, Hercules, CA, USA) according to the manufacturer’s instructions. All ddPCR reactions were prepared by mixing 12.5 μL of 2 x ddPCR supermix (Bio-Rad, Hercules, CA, USA), 1.25 μL of a 20 x primer/probe mix containing both forward and reverse primers (900 nM each, final concentration) and probe (250 nM final concentration) and a variable amount of DNA for a final volume of 25 μL. Amplification conditions on the C1000 Touch thermal cycler were as follows: one cycle at 95°C for 10 min followed by 40 cycles at 94°C for 30 s and 55.5°C for 1 min, and one cycle at 98°C for 10 min. The emulsion
droplets were kept at 4°C until they were analyzed on a QX200 droplet reader.

Data analysis
Raw data from ddPCR reactions were analyzed using QuantaSoft version 1.3.2. TableCurve 2D version 5.01 was used for curve fitting and to calculate the logistic dose-response curve. All qPCR raw data were analyzed by ViiA 7 software version 1.2.4. Statistical calculations were performed by Microsoft Excel 2016 MSO and to calculate the logistic dose-response curve. All qPCR raw data were analyzed by ViiA 7 software version 1.2.4. Statistical calculations were performed by Microsoft Excel 2016 MSO.

Data sharing statement
Novartis is committed to sharing with qualified external researchers access to patient-level data and supporting clinical documents from eligible studies. These requests are reviewed and approved by an independent review panel on the basis of scientific merit. All data provided are anonymized to respect the privacy of patients who have participated in the trial in line with applicable laws and regulations. The data availability of these trials is according to the criteria and process described on https://www.clinicalstudydatarequest.com.

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AUTHOR CONTRIBUTIONS
Conceptualization, S.T., J.L.B., C.K., and R.P.; Methodology, L.D., N.R., N.V., I.L.C., S.T., C.K., K.T.M., and R.P.; Investigation/Validation, L.D., N.R., N.V., I.L.C., S.T., K.T.M., and R.P.; Formal Analysis, L.D., N.R., N.V., and R.P.; Resources, C.K.; Data Curation, C.K.; Writing – Original Draft, L.D. and R.P.; Writing – Review & Editing, L.D., N.R., N.V., I.L.C., S.T., J.L.B., C.K., K.T.M., and R.P.; Supervision, S.T.; Project Administration, C.K.

DECLARATION OF INTERESTS
All authors are employees of Novartis Pharmaceuticals, Novartis Institutes for BioMedical Research, or Navigate BioPharma Services, a subsidiary of Novartis Pharmaceuticals.

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| Table 3. Primer and probe sequences |
|----------------------------------|
| **qPCR assay** | **Oligonucleotides** | **Sequence (5′→3′)** |
|-----------------|-------------------|------------------|
| Tisagenlecell   | forward primer    | TGC CGA TTT CCA GAA GAA GAA GAA G |
|                 | reverse primer    | GCG CTC CTC TCT AAC TCC |
| VIC TaqMan NFQ-MGB probe | ACT CTC AGT TCA CAT CGT C |
| CDKN1A forward primer | GAAAGCTGACCTGCCCTATTG |
| reverse primer | GAGAGGAATGCTTGGAGAACAT |
| 6FAM TaqMan NFQ-MGB probe | CTCGCCAGTCTCTTT |

Oligonucleotide sequences for the tisagenlecell and CDKN1A qPCR and ddPCR assays are shown. 6FAM and VIC are 5′ reporter fluorophores, and NFQ-MGB is a 3′ non-fluorescent quencher-minor groove binder. Supplemental Material CDKN1A, cyclin-dependent kinase inhibitor 1A; NFQ-MGB, non-fluorescent quencher-minor groove binder.
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