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Development of a Human DNA Quantitation System

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The AluQuant™ Human DNA Quantitation System has been developed for human-specific quantitation of forensic samples. This system uses probes specific to repetitive genetic elements allowing quantitation without target amplification. Target immobilization is unnecessary with employment of solution hybridization. The AluQuant™ Human DNA Quantitation System uses a series of enzymatic reactions to produce a luminescent signal proportional to the quantity of human DNA present. This report demonstrates a range of quantitation from 0.1-50 ng of human DNA. Signal from non-human DNAs tested was insignificant and addition of non-human DNAs into a human sample did not alter quantitation. Lastly, the system was unaffected by degradation of sample through sonication. The AluQuant™ Human DNA Quantitation System is a simple and sensitive method for quantitating the concentration of human DNA in forensic samples.

Key words: *DNA; DNA-directed DNA polymerase; forensic medicine; luciferase; luciferins; luminescence; repetitive sequences, nucleic acid*

In human forensic identification, sensitive polymerase chain reaction (PCR) multiplex assays are used for genotyping of biological samples. These genotyping assays function optimally within a narrow range of template DNA, necessitating accurate and consistent quantitation of DNA. Furthermore, forensic samples collected from a crime scene are often contaminated with bacteria and fungi. As such, contaminating DNA from other species must be disregarded in the quantitation of the human component of the total DNA. Quantitation of human DNA in a sample can be separated from possible contributions from other species through the use of DNA probes specific to human DNA. Most commonly, use of such probes often requires immobilization of the target DNA on a solid support and hybridization of probe to the DNA. This is followed by a series of washing steps at various stringencies to remove unbound probe. This process can be time consuming, produces variable results, and has a limited quantitation range.

The AluQuant™ Human DNA Quantitation System is a novel system designed for use in forensic DNA analysis, before PCR-based genotype analysis. This system is based on the READIT™ Technology from Promega (Madison, WI, USA, ref. 1). The AluQuant™ Human DNA Quantitation System allows measurement of human DNA through the use of probes to highly repeated sequences present in the chromosomal DNA without amplification of the target DNA. Such highly repeated sequences have been identified in the chromosomes of human DNA and are specific to humans (and possibly higher primates). Additional specificity is provided by the fidelity of DNA polymerase in recognition of perfect hybrids (2). Consequently, the AluQuant™ Human DNA Quantitation System has been shown to be unaffected by the presence of DNA from other species. Lastly, the AluQuant™ Human DNA Quantitation System avoids the use of gel electrophoresis or blotting steps by using a liquid chemistry. This solution hybridization approach is amenable to automation and should be more effective with degraded DNA than membrane-based hybridization systems.

In this study, we present data demonstrating the relationship of light signal to DNA concentration, evaluate the effective quantitative range, test the human specificity, and examine the effect of degraded DNA on the system. Additionally, an explanation of the theory behind the AluQuant™ Human DNA Quantitation System is presented.

Theory of the AluQuant™ Human DNA Quantitation System

The AluQuant™ Human DNA Quantitation System uses two incubation steps to measure the amount of human DNA (Fig. 1). Initially, purified genomic DNA is denatured to allow access of the human specific probe added in the first incubation step. When the sample is added to the reaction mixture, probe is allowed to hybridize to the target DNA and this initiates a series of two coupled enzymatic reactions. The

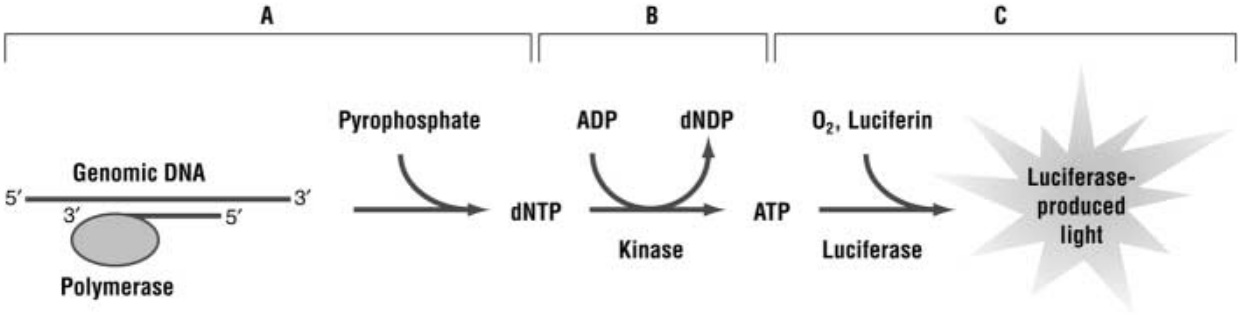

Figure 1. Chemical pathway of the AluQuant™ Human DNA Quantitation System. Section A illustrates the pyrophosphorylation reaction. Section B illustrates the transfer of the terminal phosphate group from the released dNTP to ADP by the READase™ Kinase to form ATP. Section C illustrates the production of light by luciferase and ATP.

first reaction (Fig. 1A), known as a pyrophosphorylation reaction (2), is the true reversal of the DNA polymerization reaction. During this reaction, a DNA polymerase, known as the READase™ Polymerase, catalyzes the addition of a pyrophosphate across the 3'-terminal bond of double-stranded DNA. This addition results in the release of the 3'-terminal base from the DNA strand as a deoxynucleotide triphosphate (dNTP). The terminal phosphate of the released dNTP is then transferred to adenosine diphosphate to form adenosine triphosphate (ATP) using the second enzyme in the reaction mixture, the READase™ Kinase (Fig. 1B). In the second incubation, ATP produced in the first reaction is used by luciferase (Fig. 1C) to produce a proportional and measurable amount of light (3).

In cases where the probe hybridizes to the target DNA with no mismatches (as with human DNA), the READase™ Polymerase recognizes the hybrid as a substrate for pyrophosphorylation and dNTPs are produced. However, if there are mismatched bases near the 3'-end of the probe-target DNA hybrid, the complex is not an effective substrate for the polymerase (2) and very little generation of dNTPs takes place. This is the expected result of interrogating non-human DNA. Since release of dNTPs is necessary to produce ATP and eventually light, a signal will not be produced with non-human target DNA.

Because signal in the assay is dependent on the concentration of ATP, pre-existing ATP or dNTPs in a sample will produce non-specific signal. Additionally, target DNA that reanneals to form double-stranded DNA templates with either blunt-ends or 5'-overhang ends can be recognized by the READase™ Polymerase and consequently pyrophosphorylated. This background signal in each sample is measured by concurrent analysis of a sample without human specific probe (negative control). The net signal for a sample can then be determined by subtracting the signal of the control reaction from the signal measured in the presence of probe. The quantity of DNA in a sample is then calculated through the comparison of signal from the unknown to a standard curve of the light signals produced by known DNA standards being concurrently analyzed.

Material and Methods

DNA Standard

The DNA standard was made by creating a serial dilution of human genomic DNA that is a mixture of the DNAs from several individuals (20 ng/μL) (Promega). Dilutions were made in 0.1 x TE buffer (4) and generally at 4 ng/μL, 2 ng/μL, 0.5 ng/μL, 0.1 ng/μL, 0.02 ng/μL, and 0 ng/μL concentrations. DNA standards were analyzed similar to sample DNAs, as described below.

DNA Used for Sample Analysis

Various dilutions of human genomic DNA (20 ng/μL) (Promega) were used to test the standard curve. Cross-reactivity and interference studies used yeast DNA from Promega, chicken, cow, pig, fruit fly, mouse, rabbit, and rat DNA from Clontech (Palo Alto, CA, USA), and *E. coli* (strain B), *M. lysodikticus*, and *C. perfringens* from Sigma Corporation (St. Louis, MO, USA).

Preparation of Luciferase/Luciferin Reagent

Luciferase/Luciferin (L/L) reagent was prepared by sampling 12 mL Enliten® Luciferase/Luciferin Reconstitution Buffer (Promega) into a vial of Enliten® Luciferase/Luciferin Reagent (Promega). The solution was mixed by inversion and allowed to equilibrate to room temperature for at least 60 minutes.

Preparation of Master Mixes

Two master mixes were prepared for every analysis, one master mix containing the AluQuant™ Probe Mix (Promega) and the other substituting nuclease-free water (Promega) for the AluQuant™ Probe Mix. For each sample standard being analyzed the mixes contained 5 μL AluQuant™ Neutralization Solution, 10 μL AluQuant™ Enzyme Solution (containing READase™ Polymerase and READase™ Kinase), and 5 μL AluQuant™ Probe Mix or water (Promega).

Assay Procedure

Initial denaturation was performed in duplicate by mixing 5 μL of DNA samples and standards with 5 μL Denaturation Solution (Promega) and then incubating for 10 min at room temperature. Twenty microliters of master mix with probe was added to one duplicate of each denatured sample and standard, and 20 μL of master mix without probe was added to the other duplicate. All the reactions were then incubated at 55°C for 60 min. After incubation, 25 μL from each reaction was transferred into a luminometer tube containing 50 μL of prepared L/L reagent. Light output was measured by use of a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA, USA) with a 3 s delay, 15 s integration, and 52.1% sensitivity. The unit of measure is relative light units (RLU).

Calculation of DNA Quantity

A Microsoft Excel® macro has been designed by Promega to calculate the quantity of DNA in a sample. The macro initially calculated the net light units of a sample or standard by subtracting the signal (RLU) of the reaction without probe from the signal (RLU) of the reaction with probe. The macro then created a standard curve from the DNA standard dilution data by plotting net

light units vs DNA concentration. The quantity of DNA in an unknown sample was then calculated by comparing the net light units of the unknown to this standard curve.

Results and Discussion

Relationship of Light Signal versus DNA Concentration

An example of a standard curve of the net signal produced from human DNA by use of the AluQuant™ Human DNA Quantitation System is given in Figure 2. Six replicates at 100 ng, 50 ng, 25 ng, 10 ng, 5 ng, and 0 ng DNA were assayed. The net signal for each DNA quantity (signal from sample plus probe reaction minus the signal from corresponding sample control) was averaged and plotted relative to the quantity of DNA. As illustrated, the amount of signal measured was proportional to the amount of human DNA in the sample over a wide range of DNA concentrations. Figure 3 illustrates a similar demonstration of the relationship between light output and DNA quantity in the sub-nanogram range. These data represent analyses of 1.0 ng, 0.5 ng, 0.25 ng, 0.15 ng, 0.1 ng, and 0.05 ng DNA. In general, 0.1–50 ng of human DNA consistently splayed a direct relationship between the measured signal and the amount of DNA in the sample. In general, the AluQuant™ Human DNA Quantitation System displayed minimum sensitivity at and below 0.1 ng of DNA.

Cross-reactivity with Non-Human DNA

To evaluate the specificity of the AluQuant™ Human DNA Quantitation System, numerous species of potential concern in analysis of forensic samples were tested. Minimal or no signal was detected from DNA of fruit fly, rabbit, chicken, mouse, yeast, cow, *E. coli* (strain B), *M. lysodikticus*, and *C. perfringens* relative to similar quantities of human DNA (Fig. 4). Similar results were found with dog and pig DNA (data not shown). Yeast DNA was tested at quantities as high as 100 ng and not shown to produce an increase in net signal (data not shown). These data indicate that the AluQuant™ Probe Mix was specific for human DNA and did not detect the various species of DNA here including yeast, bacteria, insect, avian, and mammal (non-primate).

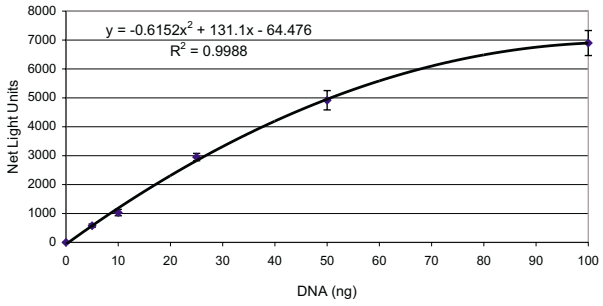

Figure 2. Effective quantitation range of the AluQuant™ Human DNA Quantitation System. The net signal produced by six replicates of 100 ng, 50 ng, 25 ng, 10 ng, 5 ng, and 0 ng DNA was measured and the average signal for each quantity of DNA was plotted relative to DNA quantity. Bars indicate one standard deviation.

In addition to testing the specificity of the system, interference of contaminating DNA in quantitating human DNA was examined. To test for interference, 2 ng of human DNA was tested in the presence of increasing amounts of non-human DNA. Figure 5 demonstrates that the signal from the human DNA in the sample remained relatively unchanged with as much as 40 ng of contaminating mouse or *E. coli* DNA. Similar tests with contaminating yeast DNA displayed minimal change in signal with as much as 200-fold higher yeast DNA (data not shown). In general, the AluQuant™ Human DNA Quantitation System was relatively insensitive to the presence of DNA from other species, even when the DNA is 20-fold higher than the human DNA. Thus the system did not give a signal with such DNAs and remained accurate in the presence of such DNAs even when the interfering DNA exceeded the human DNA in the sample.

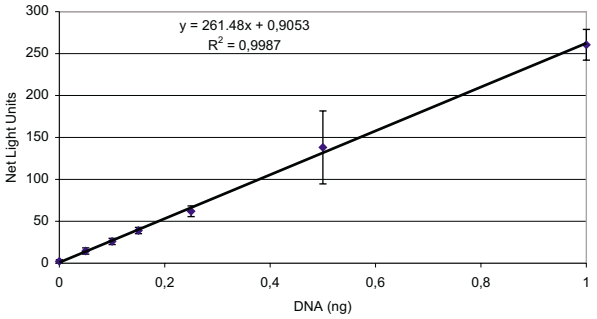

Figure 3. Sub-nanogram DNA quantitation. The net signal produced by six replicates of 1.0 ng, 0.5 ng, 0.25 ng, 0.15 ng, 0.1 ng, and 0.05 ng DNA was measured and the average signal for each quantity of DNA was plotted relative to DNA quantity. Bars indicate one standard deviation.

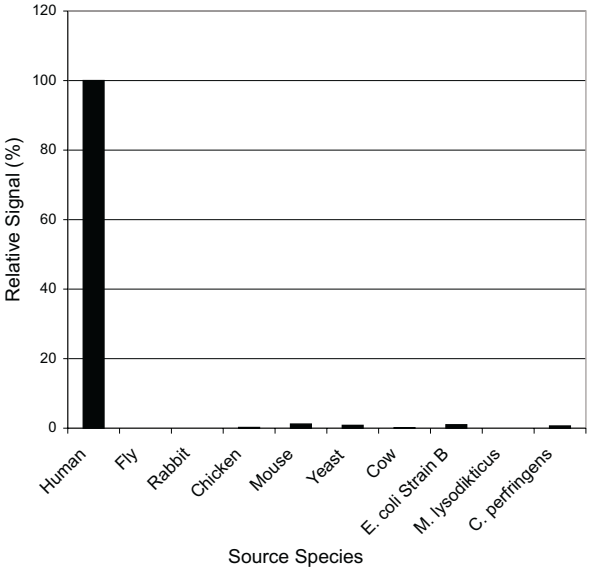

Figure 4. Reactivity with non-human DNA. Signal produced with DNA of fruit fly, rabbit, chicken, mouse, yeast, cow, *E. coli* (strain B), *M. lysodikticus*, and *C. perfringens* was compared to the signal produced from equal quantities of human DNA.

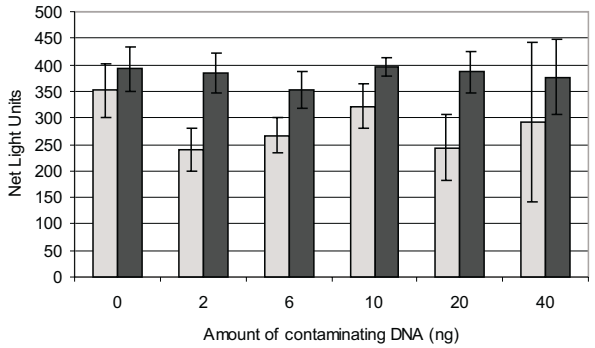

Figure 5. Non-interference of contaminating DNA. Increasing quantities of mouse and *E. coli* DNA were added to 2 ng of human DNA (performed in triplicate). Average signal for each mixture ratio was calculated and plotted. Light columns – *E. coli*; dark columns – mouse. Bars indicate one standard deviation.

Degraded DNA

A drawback of quantitation systems that require target immobilization is a reduced retention and hybridization in highly degraded samples. A potential advantage of the AluQuant™ Human DNA Quantitation System's solution hybridization format is that sensitivity should be relatively unaffected by DNA degradation. To test the effect of degradation on signal, human DNA, analyzed in triplicate, was sonicated on ice for 0, 6, and 10 min to shear the DNA. Decrease in signal was not observed with an increase in degradation from sonication (data not shown).

Conclusion

The AluQuant™ Human DNA Quantitation System has been developed as a simple and consistent method of quantitating forensic samples. The system has been demonstrated to be specific to human DNA and unaffected by contaminating non-primate DNA, even in significant excess. The human-specific probes are highly sensitive and allowed regular quantitation down to 100 pg without PCR, while maintaining a 500-fold quantitation range. Furthermore, a convenient solution hybridization format allows a blot-free, automation-friendly system. This liquid solution format also allows the AluQuant™ Human DNA Quantitation System to be relatively unaffected by degraded DNA. The numerical output of this system also increases objectivity of the human-specific DNA quantitation process. Overall, the AluQuant™ Human DNA Quantitation System provides a versatile alternative to the forensic community for quantitation of human DNA.

Acknowledgment

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