REVIEW

BIOTECHNOLOGY and BIOENGINEERING

Residual DNA Analysis in Biologics Development: Review of Measurement and Quantitation Technologies and Future Directions

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ABSTRACT: Residual DNA (rDNA) is comprised of deoxyribonucleic acid (DNA) fragments and longer length molecules originating from the host organism that may be present in samples from recombinant biological processes. Although similar in basic structural base pair units, rDNA may exist in different sizes and physical forms. Interest in measuring rDNA in recombinant products is based primarily on demonstration of effective purification during manufacturing, but also on some hypothetical concerns that, in rare cases, depending on the host expression system, some DNA sequences may be potentially infectious or oncogenic (e.g., HIV virus and the Ras oncogene, respectively). Recent studies suggest that a sequence known as long interspersed nucleotide element-1 (LINE-1), widely distributed in the mammalian genome, is active as a retrotransposon that can be transcribed to RNA, reverse-transcribed into DNA and inserts into a new site in genome. This integration process could potentially disrupt critical gene functions or induce tumorigenesis in mammals. Genomic DNA from microbial sources, on the other hand, could add to risk of immunogenicity to the target recombinant protein being expressed, due to the high CpG content and unmethylated DNA sequence. For these and other reasons, it is necessary for manufacturers to show clearance of DNA throughout production processes and to confirm low levels in the final drug substance using an appropriately specific and quantitative analytical method. The heterogeneity of potential rDNA sequences that might be makes the testing of all potential analytes challenging. The most common methodology for rDNA quantitation used currently is real-time polymerase chain reaction (RT-PCR), a robust and proven technology. Like most rDNA quantitation methods, the specificity of RT-PCR is limited by the sequences to which the primers are directed. To address this, primase-based whole genome amplification is introduced herein. This paper will review the recent advancement in rDNA quantitation and recent findings regarding potential risks of immunogenicity, infectivity, and oncogenicity of rDNA.

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KEYWORDS: residual DNA; real-time polymerase chain reaction (RT-PCR); whole genome amplification (WGA); immunogenicity; infectivity; oncogenicity

Introduction

Residual DNA (rDNA) is defined as the sum total of deoxyribonucleic acid (DNA) and fragments present in biological samples derived from recombinant host cells during expression, sometimes it is also referred to as residual host cell DNA. The potential risks associated with rDNA are infectivity (through virus such as HIV), oncogenicity (through oncogenes such as Ras), immunogenicity (through CpG rich sequences from bacteria), and mutagenesis (through transposons, retrotransposons, and DNA recombination). The potential risks associated with the presence of rDNA in products developed for human use, and a regulatory requirement to confirm its clearance in bioprocesses, is the primary reason for rDNA levels in final drug substances are closely monitored. Test methods need to be accurate, sensitive, and quantitative to assure DNA is cleared to specified levels. Throughout the history of the development of biotechnological products, methods of analysis for rDNA have evolved. Many different types of methods have been developed and are still in use by manufacturers. A review of these methods and current knowledge on the safety of rDNA is described.

Regulatory Aspects

In a 1987 World Health Organization (WHO) guideline, it was suggested that the total rDNA should not exceed 100 pg/dose (WHO, 1987). Later on, the limit was modified

to 10 ng per dose (Lebron et al., 2006; WHO, 1998), which is commonly required by regulatory agencies. This differs from host cell protein (HCP) impurities, which are handled case by case (Champion et al., 2005; Wang et al., 2009). For low dosage biologics (<1 mg/kg), a number of rDNA quantitation methods have sufficient sensitivity. However, when the dosage of biologics reaches 20–30 mg/kg or higher, it becomes a challenge to develop a robust method even with the most sensitive methods. Recent publications from both FDA and WHO provide more updated guidelines and the desire for a risk-based assessment of host cell impurities (FDA, 2010; WHO, 2010).

Risk Assessment

Since rDNA could come from a wide variety of hosts, the potential risks associated with them are also different. Furthermore, rDNA may be present in a complex array of size and composition, so risks can be difficult to generalize. Nonetheless, many aspects of DNA risk have been studied, including infectivity, oncogenicity, immunogenicity, and mutagenesis. The following sections discuss rDNA risks in more detail.

Virus Infectivity and Oncogenicity

The majority of published studies on rDNA infectivity and oncogenicity are from Peden et al. (2004) at the Food and Drug Administration (FDA). In a recent study on rDNA oncogenicity, they used both H-ras and c-myc to study tumor induction, and it was found that 12.5 μg each of plasmid containing H-ras and c-myc were needed to induce tumor growth in adult or newborn mice (Li et al., 2008). If this information was put into the context of the whole human genome, it translates to genomic DNA in the range of 1–10 g to have similar cell transformation effect because this test was using the specified gene only whereas in a genomic DNA preparation this gene is only a very small proportion of the whole genome. According to this model, which suggests very high quantities of genomic DNA for effect, the risk of rDNA for tumor induction appears to be low. Future studies using different oncogenes and animal models may expand our knowledge in this area. In a related publication, for rDNA infectivity, HIV-1 (human immunodeficiency virus type-1) was used as the transfection agent, and two separate cell lines evaluated to test infectivity (Li et al., 2009). It was found that linear DNA is more active than supercoiled DNA, and rDNA at 2 μg was infectious. These two studies suggested that virus infectivity is more likely than oncogene transformation from rDNA. However, direct comparison is confounded by the fact that the infectivity test was carried out in cell lines whereas the oncogenesis study was conducted in animals. However, taken together these studies suggest that the suggested limit of 10 ng per dose provides a significant safety factor ($>10^5$) for both infectivity and oncogenicity when taking into consideration that the tests were performed using pure oncogenes and HIV virus, respectively. An interesting model was proposed recently to address the risks of residual host cell DNA (Yang et al., 2010), it is expected that more studies will be conducted in the future to address this important issue in biologics development.

Immunogenicity of rDNA

When biologics are administered therapeutically, the response of the human immune system may be unpredictable. The "cytokine storm" induced in the clinic by TGN1412 indicated the serious consequences of unexpected mammalian responses in response to a particular biotherapeutic (Suntharalingam et al., 2006). This is believed to be target/mechanism based, but underscores the interest in de-risking biological drug candidates prior to clinical use. Protein induced immunogenicity has been published (Cavagnaro, 1995; Cohen et al., 2008; Dayan, 1995; Ryff and Schellekens, 2002; Sgro, 1995; Thomas, 1995), but much less is available regarding the capacity of rDNA to induce immunogenicity. Immune responses have been reported to high doses of specific nucleic acid sequences, for example, CpG ODNs (oligodeoxynucleotides) when present as DNA vaccine or adjuvants in preclinical and clinical studies (Barouch et al., 2003; Klinman et al., 1997; Kojima et al., 2002; Shoda et al., 2001; Stan et al., 2001; Tang et al., 2009; Verfaillie et al., 2005). The induction of anti-DNA antibodies has also been reported (Arfaj et al., 2007; Bastian et al., 1985; Gilkeson et al., 1989; Karounos et al., 1988; Pisetsky, 1997; Stollar, 1987). At some concentration, all types of DNA are expected to elicit an immune response, which depends on the source and type of DNA. These studies have demonstrated that bacterial DNA is more immunogenic than that from mammalian cells (Gilkeson et al., 1989), in part due to the high ratio of *CpG* content in bacteria and the fact that the *CpG* from bacteria is less methylated than mammalian DNA (Klinman et al., 1997). The extent of immune response in animals has been shown to be proportional to the amount of CpG in a plasmid and the ratio of CpG to all DNA present (Klinman et al., 1997; Kojima et al., 2002). Currently, high CpG content plasmids are used as adjuvant in DNA vaccine development to enhance immune response from the host (Daftarian et al., 2005; Ratanamart et al., 2007). In addition to earlier evidence showing that *CpG* ODNs are recognized by TLR9 (Kojima et al., 2002); recognition of bacterial DNA is still a matter of study and can occur by several pathways. TLR, a family of pattern recognition receptors essential for innate immunity (Kumar et al., 2009), recognizes CpG ODN by TLR9 binding. Bacterial DNA can be recognized by its sugar backbone especially the 2′-deoxyribose, through an unknown mechanism. In addition, study by Chiu et al. (2009) suggested that DNA-dependent RNA polymerase III can act as a DNA sensor linking bacterial or viral DNA to IFN-β

production and immune response, thus providing an alternative route to the TLR9 pathway for DNA-induced immune response.

To further evaluate the potential of rDNA to provoke a primary innate immune response, we have employed a human whole blood assay to test interleukin-6 (IL-6) induction in response to exposure to DNA from Chinese hamster ovary (CHO), NS0 (a myeloma cell line) and *Escherichia coli*. Production of this pro-inflammatory cytokine evaluates innate immunity, which may be a precursor to acquired immunity leading to immunogenicity. Cytokine release from the whole human blood has been used extensively to evaluate immunotoxicity of drug candidates (Groote et al., 1992; Langezaal et al., 2001; Meager, 2006; Prabhakar et al., 2002; Vial and Descotes, 1995). These types of tests have also proved useful in biologics development to evaluate the presence or confirm the absence of host impurities (Wang et al., 2009). During the evaluation of the method, it was found that the positive control material, lipopolysaccharide (LPS), needs to be at 0.02 EU/mL or lower to get a negative IL-6 response in this whole human blood assay (data not shown). Therefore, rDNA from CHO, NS0, and *E. coli* were purified and cleared of LPS with extensive extraction using LPS-removing reagent. As shown in Figure 1, for all three different DNA samples tested at 10 μg/mL, no IL-6 induction activities were detected either in the presence or absence of therapeutic proteins. In all the tests, the LPS levels were $<$0.02 EU/mL by the limulus amebocyte lysate assay.

It is noted that although a rapid response in a whole human blood assay mimics the innate response of the human immune system, it does not necessarily predict immunogenicity. IL-6 was monitored in these studies and it is likely that other cytokine responses could be induced at the levels of DNA tested; further testing with a wider cytokine panel may be warranted. Another possible explanation for the inability of fairly high levels of DNA to elicit IL-6 production in our system is the fact that nucleic acids are recognized in the endosome or cytosol instead of on the cell surface where only the receptors for lipids or protein immunogens exist (Kumar et al., 2009). It is possible that during the relatively short incubation time, an insufficient level of DNA was taken up by the cytokine-producing cells. The evidence to support this argument is in DNA vaccine development, where gene-bearing plasmids delivered with a gene gun were required to elicit an immune response in both preclinical and clinical settings (Leitner et al., 2009). In contrast, this same human whole blood assay system has been used successfully to monitor some HCPs at low levels (data not shown).

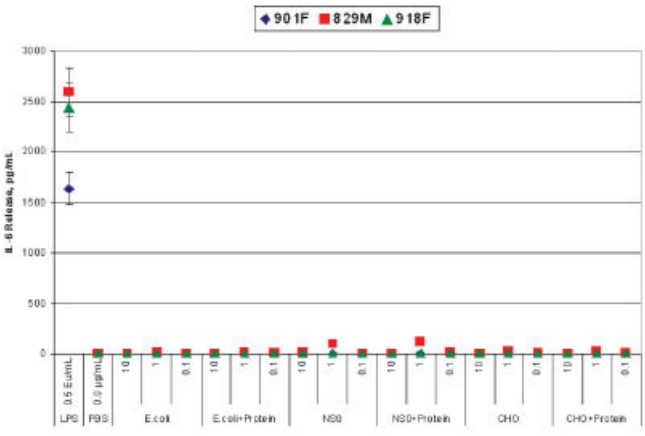

Figure 1. IL-6 release testing of genomic DNA from *E. coli*, NS0, and CHO in the human whole blood assay. Genomic DNA was added at 0.1, 1, and 10 μg/mL in the absence and presence of monoclonal antibodies. In samples where monoclonal antibodies were added, the protein concentration was at 1 mg/mL. Whole blood samples from three healthy donors (labeled 901F, 829M, and 918F, respectively) were used for the testing. The IL-6 ELISA kit from R&D System was used for the assay.

High Density Transposable Elements From Mammalian Genome

Sequencing of both the human and mouse genome indicated that mammalians have a high percentage of their genome as repetitive elements, and these repeats exist in a very high copy number and widely distributed through the mammal genome (Gibbs et al., 2004; Venter et al., 2001; Waterston et al., 2002). All mammalians have essentially the same four classes of transposable elements (Smit, 1993, 1999; Smit and Riggs, 1996; Smit et al., 1995): class I. autonomous long interspersed nucleotide element (LINE); class II. Short interspersed nucleotide element; class III. Retrovirus-like elements with terminal repeats (LTRs) and class IV. DNA transposons. The first three classes are produced by reverse transcription of an RNA intermediate (retroposition), whereas DNA transposons move by a cut-and-paste mechanism of DNA sequence. Repetitive sequences are the single most prevalent feature of mammalian genomes (Waterston et al., 2002). It is believed that these repetitive sequences are the remains of transposable elements and these transposable elements are a product of the evolution of the genome. Sequencing has revealed that at least 50% and probably more of the human genome is composed of repeat sequences and those sequences share considerable homology with the high copy repeats determined from both the rat and mouse genome (Allen et al., 2003; Boyle et al., 1990). It is recognized that as active elements, those repeats have reshaped the genome by causing ectopic rearrangements, creating entirely new genes, modifying, and reshuffling existing genes and modulating overall GC content (Venter et al., 2001). It is also known that most of the human and mouse repeat sequences are derived from transposable elements.

The risks to humans of exposure to these highly abundant repeat elements remain unknown, but if there is rDNA present in biological products, a relatively large proportion of the DNA fragments would contain repetitive sequences.

For example, if we follow the specification of 10 ng rDNA per dose proposed by WHO, and also assume that the average fragment of DNA in the final drug product is 5,000 base pairs and the average nucleotide pair has molecular weight of 660 Dalton, then 10 ng of rDNA would have 9×10^8 copies of the repetitive elements. In addition, a recent study in human neural progenitor cells indicated that de novo L1 retrotransposition events may occur in the human brain (Coufal et al., 2009), and it was suggested that this process may contribute to individual somatic mosaicism where cells show different genetic composition in the same organism. Many rDNA quantitation methods have been developed and are capable of recognizing highly repetitive sequences which have been confirmed to be present in samples derived from bioprocesses (Lovatt, 2002; Mehta and Keer, 2007; Venable et al., 2007). However, the integration of repetitive sequences in mouse DNA has not been shown to be integrated into human cell lines, so the risk remains hypothetical.

Traditional rDNA Analysis

There are several methods that have been historically used for the quantitation of rDNA in bioprocesses from recombinant DNA technology. The first one is the PicoGreen method. In this approach, a dye called PicoGreen® binds to double stranded DNA and generates fluorescence, whereas unbound dye emits little fluorescence (DiPaolo et al., 1999). The advantage of the PicoGreen® method is its ease of use and low cost, and has some utility for samples with relatively high levels of DNA (e.g., upstream bioprocess samples). However, PicoGreen® has relatively poor sensitivity and can detect only about 0.2 ng of a pure solution of DNA. Like all methods for rDNA, it is also prone to interference and from other components (including high recombinant protein) and does not give reliable results unless DNA is extracted. The lack of sensitivity is the major limitation, however, given the guideline from WHO for not more than 10 ng rDNA per dose of drug. For this reason alone the method is rarely suitable for final product release, especially high dose proteins. However, low dose proteins (e.g., vaccines), may be suitable if properly validated and PicoGreen® offers the advantage of high throughput and low cost.

Another technique used for rDNA quantitation is hybridization. In this method, probes designed to recognize specific target single stranded DNA sequences are labeled with either radioactive tags or fluorescence dyes and hybridized with denatured DNA samples immobilized on a membrane. The amount of DNA is estimated from the signal intensity relative to a DNA standard, which is generally the genomic DNA from the host (Mehta and Keer, 2007). The advantage of the technology is that it is similar to the Southern Blot widely used in molecular biology, no special training is needed, and the cost is relatively low. Another advantage is the size of the DNA can be estimated since the DNA can be electrophoresed prior to blotting alongside DNA standards of various molecular weights. Like PicoGreen®, the major drawback of this method is the lack of sensitivity (usually about 10 ng/mL). It also suffers from relatively long testing time (48 h). Because of these disadvantages, the method is no longer widely used in the biotechnology industry.

Another common method used in the biotechnology industry is Threshold® technology. In this method, a single-stranded DNA binding protein (Chase and Williams, 1986) is used to bind and capture denatured single stranded rDNA and a monoclonal anti-DNA antibody (Kung et al., 1990) conjugated to urease is used to detect the bound DNA. The hydrolysis of urea by the enzyme produces a change in pH in a small reaction chamber. The rate of change in pH is therefore proportional to the amount of DNA in samples, which are then calculated relative to signals from DNA standards. The advantage of this method is its sensitivity (1–3 pg) and standardized protocol; the disadvantage is the relative high cost and low throughput. Another point to consider for this assay is the limitation of the detectable size of DNA fragments from biologics. Because this method depends on the binding of the denatured DNA to the single-stranded DNA binding protein and the monoclonal anti-DNA molecule, DNA fragments <600 bp will be missed. During bioprocessing it is possible that a DNA will be sheared to smaller sizes during the treatment through filters and chromatography columns.

Real-Time Polymerase Chain Reaction (RT-PCR) for rDNA Analysis

Overview of RT-PCR Technology

Given the importance of rDNA testing in biologics development, a sensitive, fast, and cost-efficient method is needed. During the past several years, PCR-based technology has been used for the determination of rDNA and now becomes the most widely used method in the biotechnology industry. During the PCR reaction, a sequence-specific DNA template is amplified to produce billions of copies within 1–2 h (Fig. 2); this technology thus allows the detection of extremely low levels of DNA. The earliest PCR methods were based on an end product approach where the final PCR products were analyzed with an agarose gel. This additional processing limited the quantitative aspects of the method. A major advance was realized with technologies capable of monitoring DNA amplification in real time. (Gijsbers et al., 2005; Gregory et al., 2001; Lahijani et al., 1998; Lovatt, 2002; Lovatt et al., 2002; Smith et al., 1999; Wang et al., 2006). PCR products are measured in the early stage of amplification in RT-PCR, allowing the accurate quantitation of the rDNA in biologics products because there is a high correlation between the level of starting DNA and the signals detected in this early exponential phase. If the DNA is quantified in the linear

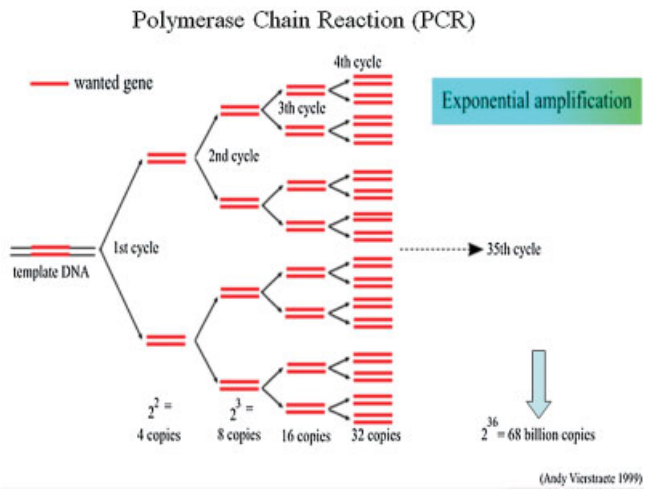

Figure 2. Diagram of the PCR and its amplification capability.

phase or in the plateau phase, the results will be highly variable (Fig. 3).

Several different chemistries are used in real-time PCR, three of them are more commonly used and they are all based on the use of fluorescent dyes. The first one is based on double-strand DNA binding dyes, such as SYBR® Green (Life Technologies, Foster City, CA). The background fluorescence from SYBR® Green as a free dye is very low when stimulated by an appropriate light wavelength. In contrast, as SYBR® Green binds to the minor groove of the double-stranded DNA generated by the PCR reaction, there is a dramatic increase in fluorescence output, about 2,000 times stronger than the free dyes, therefore this assay has a very good signal to noise ratio. The second assay chemistry is

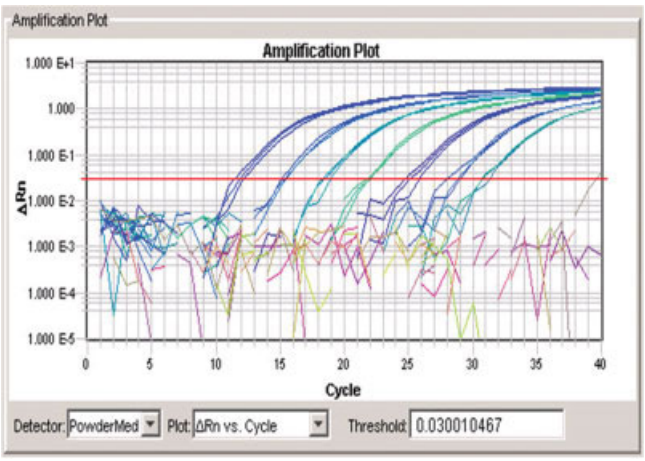

Figure 3. Amplification curve of RT-PCR on CHO genomic DNA.

based on the dye-primer based signaling systems. In this case, the fluorescence of the dye is quenched in the primer because of the special design like a hairpin. During real-time PCR, this hairpin structure is converted to single-stranded form and annealed to DNA template for extension. Ten-fold increases in fluorescence could be observed from the hairpin form to the linear form of the primer. The advantage of this method is its multiplexing capability; several genes could be monitored simultaneously. The third assay chemistry is based on TaqMan® technology. In this case, a fluorescence-labeled probe with a quencher molecule in the same fragment is designed between two sequence-specific primers. During PCR, the exonuclease activity of the polymerase will cleave the probe, release the fluorescence dye from the quencher, thus result in significant increase in fluorescence, similar to the SYBR® Green technology, TaqMan® has a signal to noise ratio above 1,000, making it a very sensitive detection method. Because our effort in developing the quantitative rDNA analysis was using the TaqMan® technology, a widely used quantitative PCR in the industry, more details were provided in the following sections.

Selection of DNA Sequences for PCR Amplification

Since the TaqMan® technology depends on the amplification of a defined DNA sequence, the first task in developing a real-time PCR method for rDNA quantitation is to determine which sequence to be used for the amplification. Therefore, methods that can sensitively detect and quantify rDNA need to be evaluated. In regards to the real-time PCR technology, it is known that the initial copy number of target DNA sequences is proportional to shorter times to achieve a threshold signal. Therefore, it is advantageous to select DNA sequences with as many copies as possible in the genome for amplification. In the following section, the discussion of the selection of specific DNA sequences is confined to *E. coli*, NS0, and CHO, three of the most commonly used hosts for production of biological products.

For *E. coli* rDNA quantitation, the most common DNA sequences are the ribosomal RNA genes because each *E. coli* genome contains seven copies each of the 5S, 16S, and 23S ribosomal RNA genes (Gregory et al., 2001; Smith et al., 1999; Wang et al., 2006) and they share very similar sequences (Fig. 4). In our initial evaluation studies, both the 5S and 16S ribosomal RNA gene sequences were tested for DNA amplification, and eventually we selected a DNA sequence of the 16S gene as a target for the RT-PCR amplification (Fig. 5). In our laboratory, 0.1 pg/mL of *E. coli* DNA could be quantified using real-time PCR (Fig. 5), this is at least 10 times more sensitive than immune assay or hybridization described earlier. It should be pointed out that the successful development of a sensitive real-time PCR for *E. coli* rDNA quantitation depends on the purification of high quality DNA as standards (Marmur, 1961).

Figure 4. Sequence alignment of the seven *E. coli* 16S ribosomal RNA gene sequence and the design of the amplicon for RT-PCR.

For mammalian cell rDNA analysis, the selection of DNA sequence for amplification was based on an analysis of rat and mouse genomes. Several families of interspersed repeats were found to be widely distributed in the mouse genome and nearly 500,000 copies (Gibbs et al., 2004; Waterston et al., 2002). Five families of those high copy elements were evaluated: LINE-1, LINE-2, B1, B2, and LTR. After alignment of the top 10 most homologous sequences in each of the aforementioned family, it becomes apparent that one family of repeats was most conserved in the mouse genome among these five families. Primers and probes were designed to recognize these sequences. It is noted that these repetitive sequences are not as highly conserved as those for the *E. coli* ribosomal RNA genes. To address these differences, two probes were designed to increase the coverage of this repetitive element family in the mouse genome. Using this design, it was found that as low as 0.01 pg/mL of genomic DNA could be quantified with real-time PCR (Fig. 6). Overall, four amplicons (the fragment that is amplified by Q-PCR) were designed and one of them showed the highest sensitivity in the real-time PCR detection. Both NS0 and CHO-derived genomic DNA were used to test the range of detection, and it was found that the same amplicon provides the highest sensitivity for both species of genomic DNA (Table I). In our further robust testing, it was shown that both the primers and probes were stable for more than 2 years without noticeable decrease of sensitivity if stored at -20°C, and the genomic DNA prepared were also stable if kept at -80°C.

Comparison of RT-PCR With Traditional DNA Quantitation Methods

Since the threshold method was used for the testing of internal biologics under development, the transition of testing method needs a careful bridging study. It was shown from multiple bioprocesses that these two methods provide parallel results at the upstream of biologics purification when the rDNA level is relatively high. However, in samples from several different stages of purification during bioprocessing, the rDNA levels is not detectable using Threshold™ yet can be quantified using RT-PCR (Fig. 7). This is due to the 10× improvement in sensitivity. These data suggest that RT-PCR has comparable coverage of rDNA species as compared to the traditional method but with increased sensitivity. In light of the regulatory agency's guideline on the rDNA levels from biologics ($\leq$10 ng/dose), the sensitivity of RT-PCR provides greater opportunity to measure at this level and achieve this target for high dose therapeutics. RT-PCR has been validated

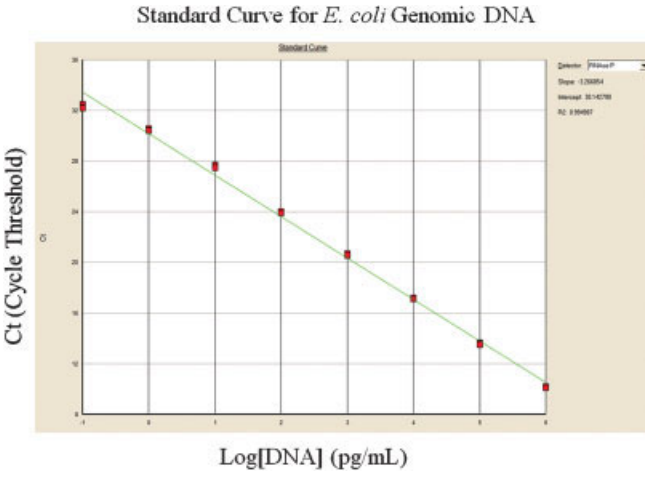

Figure 5. Standard curve of *E. coli* genomic DNA amplified with RT-PCR.

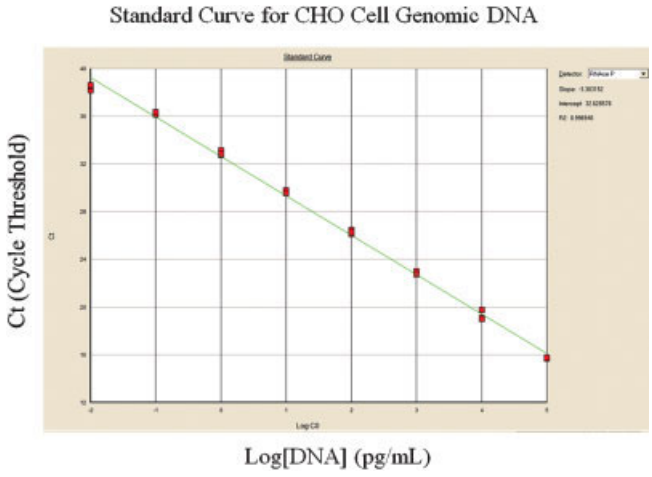

Figure 6. Standard curve for CHO genomic DNA amplified with RT-PCR.

Table I. Evaluation of different primer and probe pairs for Q-PCR of NS0 and CHO DNA.

Elements	Q-PCR sensitivity, NS0	Q-PCR sensitivity, CHO
B1 mm	++++	++++
B2 mm	+++	+++
L1 mm	+++	–
LTR	+++	–

successfully according to the ICH guidelines for many late stage projects.

Whole Genome Amplification for rDNA Analysis

Overview of pWGA Technology

As previously described, detection of DNA can be achieved by hybridization-based DNA detection, immune-detection or real-time PCR-based quantification, but all have drawbacks. PCR-based detection is based on specificity for unique representative sequences that do not represent the entire genome; hence other sequences may not be detected if not inclusive of the designated target sequences. This may be important considering that bioprocesses can cause shearing of DNA to smaller fragments and the PCR can only detect rDNA if the target DNA sequences are present. Although hybridization-based and antibody/affinity DNA detection is designed to detect all potential DNA sequences that might be present, the sensitivity of this technology is limited since, unlike PCR, the DNA is not amplified. There are sequence size restrictions for the antibody/affinity technology offered by Threshold®. Alternative technologies have been investigated, one of which is the whole genome amplification (WGA) technology (Barker et al., 2004; Gribble et al., 2004; Hosono et al., 2003; Thorstenson et al., 1998). The WGA technologies use a different mode of amplification with unique primers designed to limit the specificity. Like PCR, all WGA approaches amplify small amounts of DNA to achieve the required sensitivity. Although several WGA systems have been marketed, we are not aware of any that is currently in use for rDNA testing in biologics.

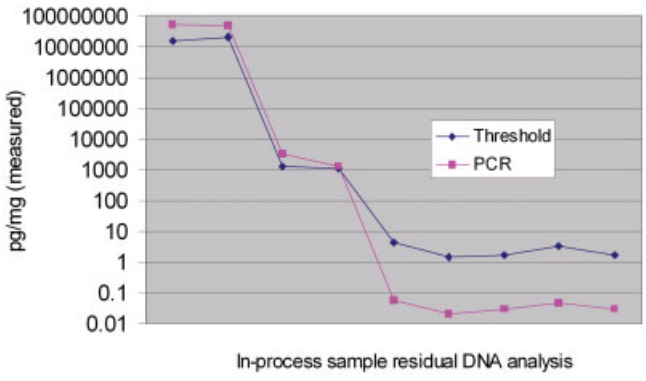

Figure 7. Comparison between threshold and RT-PCR for the determination of rDNA from typical in-process samples.

The Mechanism of WGA Technologies

The first WGA system developed was a PCR-based DNA amplification system in which specific DNA sequences were added to the ends of all DNA fragments by DNA ligase and used as the origins for amplification by PCR (Barker et al., 2004; Gribble et al., 2004; Thorstenson et al., 1998) By these methods, all DNA fragments are amplified by PCR using specific primers that bind to the added DNA sequences, when amplification was performed in real time quantification setting, the rDNA can be detected without sequence preference or selectivity. Therefore, this WGA system provides higher selectivity and sensitivity in detecting rDNA from biologics than standard PCR technology. The limitation of this technology is the effectiveness in adding specific DNA sequences to all DNA fragments since the DNA fragments without these sequences will not be amplified by PCR protocol (and therefore not detected). The Sigma–Aldrich's GenomePlex® (Sigma-Aldrich, St. Louis, MO) Complete WGA Kit is one example of this system that is commercially available.

The second type of WGA system is designed to overcome the DNA sequence requirement of the PCR technology using degenerated (lower stringency) DNA primers (Barker et al., 2004; Hosono et al., 2003). Since degenerated primers can theoretically bind to a greater number of total DNA sequences than higher stringency primers, it provides relatively high uniform DNA amplification across the entire genome with minimal sequence bias. In addition, this technology may also benefit the rDNA detection through overcoming DNA sequence preference encountered sometimes in the standard PCR protocol. In this system, degenerated DNA primers, usually a hexamer, are used to bind to DNA target(s), and therefore, broader coverage will be achieved in comparison to that with specific primers. The REPLI-g kit system developed by Qiagen Inc. is one example of this system. The REPLI-g kit contains a degenerated hexamer and a ϕ29 polymerase for amplification. The presence of hexamer in the amplification system theoretically enables any random DNA sequence in the reaction to be amplified and the strand displacement activity of ϕ29 DNA polymerase also allows amplification at isothermal condition such as 30°C, which minimizes DNA fragmentation relative to the much higher temperatures required for standard PCR. In addition, the ϕ29 DNA polymerase also carries a $3' \rightarrow 5'$ exonuclease activity that enables proofreading capacity for more accurate amplification of target DNA. The drawback of this system is that some sequence binding preference may exist for the degenerate primer that could result in amplification of certain DNA sequence over other sequences. However, the ability to provide more uniform DNA amplification from the entire genome with

minimal sequence bias still makes this a very attractive technology for rDNA detection.

The third type of the WGA system is based on the *E. coli* DNA replication system. In this system, primase is used to replace the Taq DNA polymerase. A DNA helicase is also utilized. The presence of the primase eliminates any primer requirement for initiation of DNA amplification. The presence of a DNA helicase enables double-stranded DNA targets to unwind at 37°C so that the DNA amplification can also take place at the 37°C. In addition, the presence of primase also allows multiple initiation sites for more effective amplification of target DNA. The Rapisome™ pWGA, developed by BioHelix Inc. (Beverly, MA) is one example of this type of WGA system (Fig. 8). The Rapisome™ pWGA system, in combination with the real time DNA quantification technology, provides an attractive methodology for real time detection of rDNA with high sensitivity and efficiency.

Evaluation of the pWGA System for Detection of Low Level rDNA in Biologics

Because of the potential of the Rapisome™ pWGA system in amplifying DNA with high efficiency, we have evaluated the feasibility of using the Rapisome™ pWGA system for detecting rDNA in low levels in biologics samples. First, we tested the capability of the pWGA system to amplify low levels of DNA. The human genomic DNA was diluted from 1 μg/mL to 1 pg/mL and 5 μL of the diluted DNA was incubated with 20 μL of the Rapisome™ pWGA mix at 37°C for 2 h. The amplified DNA samples were analyzed by agarose gel electrophoresis using a 0.8% gel (Fig. 9). The pWGA system effectively detected the human genomic DNA with the DNA in concentrations as low as 1 pg/mL in 2 h incubation time. To further test its feasibility for real time quantification of rDNA in biologics, we further adapted the Rapisome™ pWGA system for real time amplification of the human DNA using the ABI 7500 RT-PCR system (Life Technologies, Carlsbad, CA) (Fig. 10). The EvaGreen™ dye was used with the Rapisome™ pWGA system for effective detection of amplified DNA in the real time DNA quantification, the reason to use this dye instead of SYBR is that EvaGreen™ could provide a stronger signal thus increase the sensitivity of the detection. The human DNA

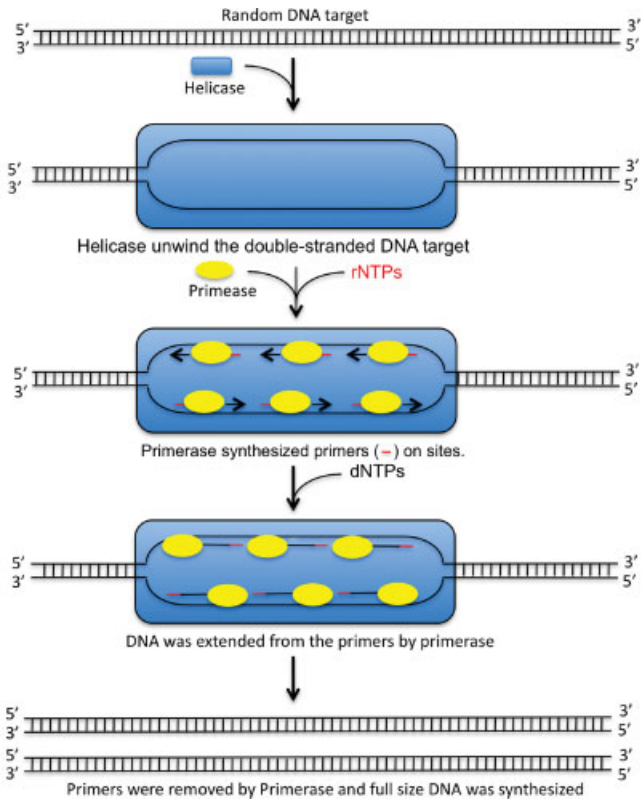

Figure 8. The model of whole genome amplification by the Rapidsome™ pWGA system.

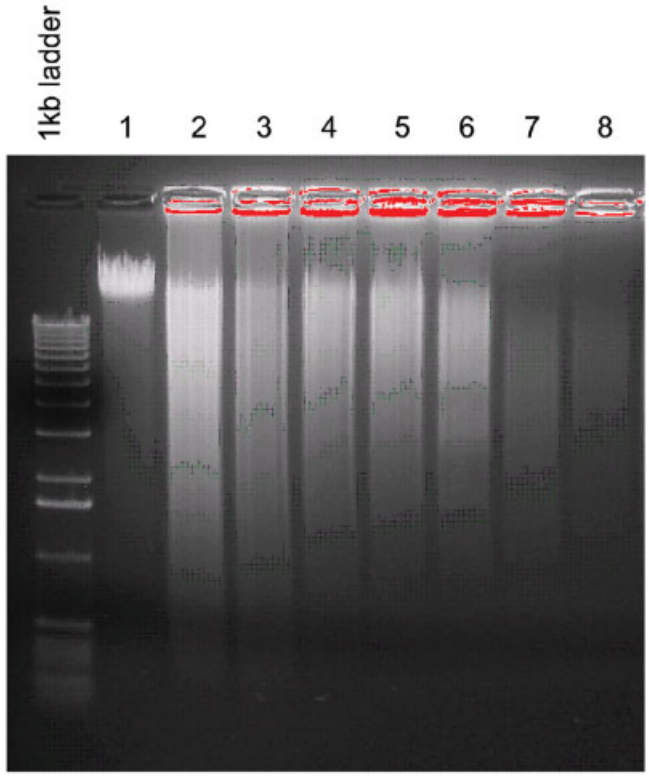

Figure 9. Detection of human genomic DNA amplified by the Rapidsome™ pWGA system using agarose gel. The human genomic DNA was diluted in TE buffer. The indicated amount of genomic DNA was incubated with 20 μl Rapidsome™ pWGA mix at 37°C for 2 h. The reactants were analyzed by agarose gel electrophoresis using a 0.8% gel. The DNA was visualized under UV light and documented by a Kodak DC290 gel documentation system. Lane 1, 2 μg human genomic DNA; lane 2, 1 ng human genomic DNA amplified by the pWGA system; lane 3, 100 pg human genomic DNA amplified by the pWGA system; lane 4, 10 pg genomic DNA amplified by the pWGA system; lane 5, 1 pg human genomic DNA amplified by the pWGA system; lane 6, 100 fg mouse genomic DNA amplified by the pWGA system; lane 7, 10 fg human genomic DNA amplified by the pWGA system; and lane 8, 1 fg human genomic DNA amplified by the pWGA system.

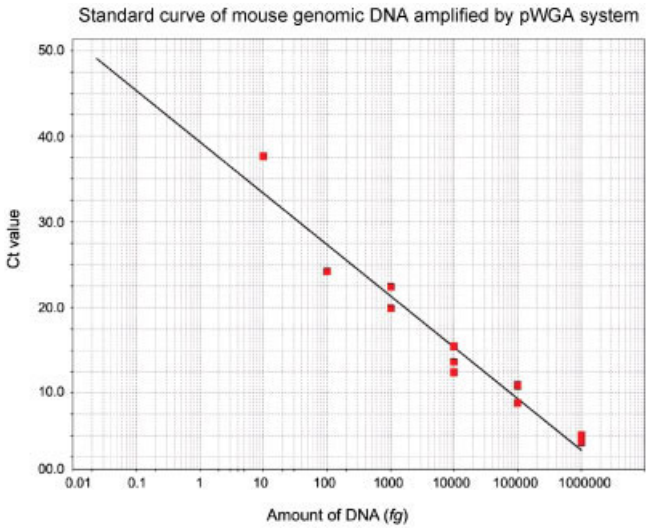

Figure 10. The Ct value standard curve of human genomic DNA generated by the Rapidsome™ pWGA system using quantitative PCR assay. The human genomic DNA was diluted into various concentrations. The indicated amount of mouse genomic DNA was then mixed with 17 μl Rapidsome™ pWGA mix and EvaGreen dye (0.6 μM of final concentration) in a total volume of 20 μl in 96-well plate. Each DNA sample was set as triplicates. The 96-well plate was then placed in an ABI 7500 RT-PCR system with the following setting: 60 cycles of 37°C for 1 min and 36°C for 1 min. The mean Ct value and the standard deviation was obtained for each DNA sample. The standard curve was generated using Ct value versus log DNA amount (fg).

was diluted into a series of dilution (1 μg/mL ~ 1 pg/mL) and was incubated with the Rapisome™ pWGA master mix in order to detect the level of DNA by the ABI 7500 Real time quantification system (Fig. 10). Because the reaction is processed in an isothermal temperature, the real time quantification of the DNA was done with 50 cycles of 36°C for 1 min and 37°C for 1 min and the threshold was determined for each DNA samples. The standard curve was generated from the threshold value of the diluted DNA samples. The results obtained from our experiments revealed a linear correlation between the level of DNA and the Ct value of the DNA in the reaction from the concentrations ranges of 1–10 pg/mL. Given the ≤10 ng rDNA/per dose of biologics recommended by the WHO's guideline, the sensitivity of the pWGA system in detecting rDNA is well below the limits of the suggested rDNA level in most doses of biologics. Therefore, the pWGA system provides an attractive system for effective measurement of low levels of rDNA in the biologics. Although more studies are needed to determine the feasibility of the pWGA system in detecting rDNA from biologics, the results of these preliminary studies provide strong evidence to suggest the possibility of applying the pWGA system to DNA detection for effective detection of rDNA from biologics.

Conclusions

The detection and quantitation of rDNA is required for all biologics in development, and high dose drugs can be challenging to test with adequate sensitivity. Similar to the analysis of HCPs, rDNA is treated as a host related impurity, and demonstration of clearance in bioprocesses to acceptable and/or consistent levels is a major parameter in biologics development. Of the theoretical rDNA risks discussed here, a knowledge gap still exist to evaluate the potential risk of the insertional mutation induced by the transposable retrotransposons that are widely present in the mouse genome. For other risks, there are limited means for evaluation, and future studies may elucidate these aspects. Until then, detection and monitoring of rDNA will be paramount in the recombinant DNA field.

Different from HCPs, where a vast heterogeneity in both size and charge makes monitoring and clearance highly challenging, DNA molecules have similar basic structure and highly negative charge. Exploitation of this property with chromatographic and charge-based separation allows clearance of rDNA several orders of magnitude more efficiently than that of HCPs. However, it is still possible that certain biologics or a certain isoforms of particular recombinant proteins, including those with net positive charge, possess a strong affinity to DNA, similar to the DNA binding proteins like zinc finger and leucine zipper (Ellenberger et al., 1992; Theunissen et al., 1992) that could make the clearance of rDNA relatively difficult. In our experience with dozens of different biologics processes, rDNA has been cleared to parts per billion levels or less. The detection and quantitation of rDNA has evolved significantly over the last decade. The DNA hybridization and immunoaffinity-based Threshold™ method provided valuable information in the early days of biologics development, whereas more recently RT-PCR has gained prevalence because of its higher sensitivity, throughput and lower cost. Based on the recent studies using whole genome amplification, it is now possible that this novel technology may prove ideal for the analysis of rDNA due to its sensitivity similar to RT-PCR, improved sequence coverage, however more studies need to be done in this area before the adaptation of this technology for rDNA analysis in biologics.

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