⟨1130⟩ NUCLEIC ACID-BASED TECHNIQUES—APPROACHES FOR DETECTING TRACE NUCLEIC ACIDS (RESIDUAL DNA TESTING)

INTRODUCTION

The basic principles of nucleic acid amplification technologies (NAT) and definitions of the various techniques are covered in *Nucleic Acid-Based Techniques—General* ⟨1125⟩. This chapter covers the analytical procedures used to quantify residual DNA in biopharmaceuticals.

Process characterization and the theoretical safety concerns associated with process-related impurities highlight the need for residual DNA testing in biopharmaceutical products. The ability of a manufacturing process to remove residual DNA from a biopharmaceutical product is an indicator of the quality and consistency of the process and that the process is under control. Additionally, the cells used to produce a biopharmaceutical can be sources of a range of complex, heterogeneous, and potentially unsafe impurities, with host cell DNA among these impurities. For continuous cell lines, the potential risk of residual DNA arises from both of its biological activities, namely infectivity and oncogenicity. Infectivity could be due to the presence of an infectious viral genome in the cellular DNA of the cell substrate. The oncogenicity activity of residual DNA could arise through its capacity to induce a normal cell to become transformed, which may lead to tumorigenicity. Although animal testing has shown that extraneous DNA can cause tumors or infections, no reports to date have demonstrated this risk in humans. Residual DNA content, up to 10 ng of residual DNA per parenteral dose, may be considered for DNA originating from mammalian cell cultures, but the acceptable residual DNA content may vary depending on the source of the residual DNA and the route of administration of the product. One can address residual DNA in biopharmaceutical processes in two ways: 1) by validating clearance during process validation; and/or 2) by monitoring residual DNA levels through routine testing of the drug substance. Generally, 10 ng per dose is the accepted limit, by health authorities, of host residual DNA derived from mammalian cell cultures. The level of concern regarding residual DNA can be tied to its source and the route of administration, so the residual DNA specification and procedure for monitoring DNA clearance for a given product should be developed in consultation with regulatory agencies. Regardless of whether routine testing of a drug substance is used to determine residual DNA content or whether DNA clearance is demonstrated by process validation, analytical procedures for the quantification of residual DNA are required. DNA amplification techniques, such as quantitative PCR (qPCR), are used most often for residual DNA testing because of their superior sensitivity and unique advantages (e.g., high specificity). The expectation is that the analytical procedure used to quantify residual DNA in biopharmaceuticals has a detection limit well below the DNA level allowed by regulators for biopharmaceuticals (often 10 ng/dose). Assays based on hybridization, DNA-binding protein, and qPCR are typically the techniques of choice because they can meet the sensitivity expectation.

SAMPLE PRETREATMENT

Analysis of residual DNA requires accurate quantification of picogram levels of DNA in mg (or larger) quantities of product, which may be in a variety of matrices. In certain circumstances, the sample can be analyzed neat in the analytical procedure with acceptable recovery and precision. When the product or other sample components interfere with the assay sample, dilution may be all that is required to overcome the interference, so long as the specified DNA content of the sample remains within the useful range of the analytical procedure. When sample dilution is not effective in reducing assay interference, it may be necessary to use more extensive sample pretreatment procedures, such as proteolytic digestion, chemical dissociation, or extractions. It may be necessary to use a combination of different pretreatment steps to remove interference to an acceptable level. Extensive sample manipulation can lead to losses of DNA or introduction of environmental DNA, and should be a consideration when using one or more sample pretreatment steps. Contamination with environmental DNA may only be a concern when using a residual DNA procedure that is not sequence specific.

Protein samples may only require digestion with proteinase (e.g., Proteinase K, Pronase) to allow the analytical method to quantitatively recover the residual DNA. It may also be possible that the DNA is bound to the sample components, and chemical dissociation (e.g., detergents) may disrupt the binding, allowing sufficient recovery in the residual DNA assay. Residual DNA test procedures often use protein reagents and the use of a chemical dissociation reagent. These materials must be used at a sufficiently low level or removed so that the analysis is not compromised.

It may be necessary to extract the DNA from the sample to remove the inhibitory substances that are causing the reduced DNA recovery. Extraction procedures are typically based on precipitating the DNA from the sample or DNA-specific binding to a matrix (e.g., magnetic beads). Historically, extraction methods based on phenol and chloroform, followed by ethanol precipitation, have been applied to the purification of DNA in molecular biology research. The phenol/chloroform extraction technique may be a useful pretreatment for residual DNA samples before analysis, but the phenol/chloroform extraction technique might not be the best choice for the low levels of DNA typically found in biopharmaceutical samples. Because of these low levels, quantitative DNA recovery with ethanol precipitation may be difficult. For this reason, a carrier molecule (e.g., glycogen) may be necessary to aid in DNA recovery if the phenol/chloroform extraction technique is used. Commercial kits are available and have been used successfully for pretreatment of residual DNA samples for improved recovery in the residual DNA assay. For example, some kits use a chaotrope (sodium iodide) and a detergent (sodium *N*-lauroyl sarcosinate) to disrupt the association of the DNA with the sample. The DNA is then co-precipitated using glycogen as the carrier molecule in the presence of isopropanol. Extraction of DNA from the sample, based on binding to a solid matrix, can be found in various formats. One of the most popular formats uses magnetic beads, where the beads are added to a sample with a binding solution to capture the sample DNA on the beads. The beads are then captured and held in the sample tube using a magnetic stand while the supernatant containing the interfering substances is removed and discarded. The beads are washed repeatedly using a magnetic

stand and a wash solution. Finally, the DNA is eluted from the beads for the assay using an elution buffer, with the beads being removed from the sample preparation using the magnetic stand.

The sample manipulation involved with pretreatment may reduce the recovery of the residual DNA or introduce environmental DNA into the sample. Great care must be taken during any sample manipulations to avoid DNA losses or contamination. The addition of target DNA-spiked samples in the residual DNA assay is a common practice. The target DNA-spiked sample should not be confused with the internal positive control (IPC), which is typically a nontarget DNA added after the sample pretreatment step to detect the presence of PCR inhibitors and to evaluate DNA amplification during the analysis. The IPC may also be introduced before the extraction to improve the control of this step. A recovery of 50%–150% of the spiked target DNA is often applied to residual DNA assays to ensure that the assay yields acceptable results. When sample characteristics (e.g., matrix effects or sample preparation method) make achieving a recovery acceptance criterion of 50%–150% impractical, then correcting the observed DNA concentration by using the load recovery percentage is also an acceptable approach.

HYBRIDIZATION-BASED RESIDUAL DNA ASSAY

The first residual DNA assays were based on DNA hybridization, wherein a DNA probe created from host cell DNA detects and quantifies the amount of complementary DNA present in the product under assay. Double-stranded host cell DNA consists of two complementary strands of DNA that are held together by hydrogen bonding. The double-stranded DNA in the test sample is denatured to single strands and immobilized to a membrane, typically a nitrocellulose or nylon membrane. The sample is probed using host cell DNA that has been denatured and labeled. The host cell DNA probe is not a specific sequence but is prepared by a random labeling procedure during which a radioactive or fluorescent label is introduced into the host cell DNA to produce the probe. When the denatured, labeled DNA probe is brought into contact with the membrane-immobilized DNA, the probe will bind to complementary sequences of the host cell DNA. If the probe is radioactive, the membrane is placed against autoradiography film for a sufficient length of time, the film is developed, and a dark spot will be observed where the test DNA was immobilized. The level of hybridization can be measured using a phosphor-imaging system. If the probe has a fluorescent label, the intensity of the spots is determined using a fluorescence-imaging system. The intensity of the spot is proportional to the amount of probe that was hybridized to the test DNA and therefore is proportional to the amount of residual DNA in the sample. The intensity of the spot can be compared visually with the intensity of spots that correspond to a standard curve yielding semiquantitative results (i.e., visual quantitation), or the intensity can be determined using an instrument (e.g., densitometer) to create a quantitative value that is compared with the values obtained from the standard curve.

DNA-BINDING PROTEIN-BASED RESIDUAL DNA ASSAY

Instrumentation is commercially available for the quantitation of residual DNA in biopharmaceuticals. The instrumentation requires reagents that use DNA-binding protein and antibodies targeted for DNA in a four-step analytical procedure.

1. The first step requires that the DNA be denatured into single-stranded DNA by sample heating. The denatured DNA is mixed with a single reagent that contains DNA-binding protein that is conjugated with streptavidin and a monoclonal anti-DNA antibody that is conjugated to urease. The DNA-binding protein and the monoclonal antibody are specific for single-stranded DNA but do not have any sequence specificity. This liquid phase facilitates the formation of reaction complexes that contain DNA, streptavidin, and urease.
2. During the second step, the sample is filtered through a biotinylated membrane that binds to the streptavidin and captures the complexes on the membrane, which is washed to remove any reagents that are not bound to the membrane.
3. During the third step, the membrane is inserted into a sensor on the instrument, where the urease in the DNA complex reacts with a urea solution in the sensor, producing ammonia and a change in pH that is detected using a light-addressable potentiometric sensor (LAPS). The change in pH directly correlates with the amount of DNA in the sample.
4. In the fourth step, the raw data from the instrument are analyzed using the appropriate software to determine the residual DNA content of the sample.

POLYMERASE CHAIN REACTION TECHNIQUES

Real-time qPCR is a procedure that is well adapted to fast sample throughput and has applications in many areas of biopharmaceutical manufacture (e.g., copy number detection, virus detection). The technique can quantify the amount of a nucleic acid target sequence in DNA from a variety of samples. The DNA probe and primers used in the analysis are key to the procedure. The most common qPCR method for detection of this amplification is referred to as the 5′ nuclease assay. In this format, the probe has a reporter dye attached to one end and a quencher dye attached to the other end. A pair of DNA primers is also added to the reaction. During the amplification reaction, a thermostable DNA polymerase initiates DNA synthesis where the DNA primer binds to the single-stranded sample (template) DNA and moves along the sample DNA, synthesizing new complementary DNA. While following the template DNA, DNA polymerase I cleaves any complementary DNA in the path. If DNA polymerase I encounters the labeled DNA probe, DNA polymerase I will cleave the probe. The reporter dye is released into the solution and, in the absence of the quencher dye, the resulting fluorescence is measured. Repeating the reaction cycle results in an amplification of the fluorescence signal. The number of cycles required for the fluorescence measurement to exceed a threshold value correlates to the amount of starting residual DNA in the sample. By comparing the fluorescence obtained from a sample to a standard curve, analysts can quantify the residual DNA in the sample.

Alternative Detection Strategies

A number of innovative detection strategies have been developed and commercialized beyond that described above. A few of the most common are as follows:

1. Intercalating cyanine dyes fluoresce after binding to double-stranded DNA. The amount of dye incorporated is proportional to the amount of target amplicon generated. These dyes are inexpensive and easy to use. The disadvantage of this technique is the lack of a specific probe to confer sequence specificity beyond that afforded by the primers, and the dye will also bind somewhat to single-stranded DNA and RNA molecules. Consequently, primer dimers or nonspecific products may affect the quantification. However, it is possible to check for the specificity of the system by running a melting curve at the end of the PCR run, based on the principle that every product has a different dissociation temperature and depending on the size and base content.
2. Other sensitive probes exist that contain a stem-loop structure with a fluorophore and a quencher at their 5′ and 3′ ends, respectively. The stem is usually six bases long, mainly consisting of cytosines and guanines, and holds the probe in the hairpin configuration. The "stem" sequence keeps the fluorophore and the quencher in close proximity, but only in the absence of a sequence complementary to the "loop" sequence. In the presence of a complementary sequence, the probe unfolds and hybridizes to the target, leading to separation between the fluorophore and the quencher, and the probe fluoresces. The amount of signal is proportional to the amount of target amplicon sequence. The increase in fluorescence that occurs is reversible, because there is no cleavage of the probe. It is also possible to design the stem structure to add specificity to this type of probe. However, these probes are often expensive, and the signal can be weak due to the limited possible physical separation between the fluorophore and the quencher.
3. A variation on the second example described above uses a single-stranded nucleic acid sequence containing the specific PCR primers, the specific probe with a stem-loop tail separating a fluorophore and a quencher, and a blocking group. The stem-loop tail is separated from the PCR primer sequence by a "PCR blocker", a chemical modification that prevents the polymerase from copying the stem-loop sequence of the primer. After extension of the primer during PCR amplification, the specific probe sequence is able to bind to its complement within the same strand of DNA. This hybridization event opens the hairpin loop so that fluorescence is no longer quenched, and an increase in signal is observed. Covalent attachment of the probe to the target amplicon ensures that each probe has a target in close proximity. Enzymatic cleavage is not required, nor a separate probe hybridization step, thereby reducing the time needed for signaling. This can result in stronger signals and lower background with faster cycling; however, these probes can be quite expensive and complicated to design.
4. Fluorogenic minor groove binder probes are short linear probes that have a minor groove binder with a nonfluorescent quencher on the 5′ end and a fluorophore on the 3′ end. The minor groove binder prevents the exonuclease activity of the DNA polymerase from cleaving the probe. Quenching occurs when the random coiling of the probe in the free form brings the quencher and the fluorophore close together. The probe is elongated when bound to its target and quenching is decreased, leading to an increase in fluorescent signal proportional to the amount of accumulated amplicon. These probes are also expensive and can produce a low signal-to-noise ratio.

Quantitative Multiplex PCR-Based Residual DNA Assay

An evolution of qPCR is quantitative multiplex PCR, where several pairs of primers and the corresponding probes are introduced in the reaction medium to simultaneously detect multiple targets. Benefits include higher throughput and better control of false-negative results, whereas disadvantages come from amplification and detection interferences, as outlined in *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩. One of the applications of this technique is a duplex qPCR, where the introduction of an exogenous DNA, called IPC (internal positive control, see *Sample Pretreatment* above), enhances confidence in the accuracy of the analysis when appropriately amplified. Multiplex qPCR is not used as often as single-target qPCR to assay host cell residual DNA in biopharmaceuticals.

RESIDUAL DNA TESTING POINTS TO CONSIDER

When developing a residual DNA assay, one should consider how the assay will be used, the structure of the DNA available (e.g., fragment length), and regulatory issues. The cost of analysis can be significant and should be considered when evaluating an assay format. In addition, environmental, health, and safety aspects should be considered. Traditionally, hybridization assays were performed using phosphorus (^{32}P)-labeled DNA and autoradiography. Because ^{32}P decays quickly, probes prepared with ^{32}P have a limited shelf life, and the precautions necessary for handling radioactive material can be cumbersome. These issues with ^{32}P labeling may make fluorescence labeling of the hybridization probe a more desirable option. If the hybridization assay is assessed visually, this process represents a semiquantitative assay, but if the intensity of the spots is determined using a densitometer or other imaging system, the results may be quantitative. DNA-binding protein assays and qPCR give quantitative results. Quantitative assays are typically preferred instead of semiquantitative assays (e.g., older hybridization-based methods), because the results are considered more accurate and precise, which allows better process monitoring and control. Because of sample matrix interference, a sample pretreatment step is often required to obtain accurate and reproducible results. Pretreatment steps can influence the recovery of DNA, so it is usually necessary to design the assay with a spike-recovery control with an acceptance criterion to ensure assay performance.

In-house controls are usually prepared in the laboratory and qualified by ultraviolet spectroscopy, using standard techniques employed in molecular biology, to determine the DNA content and purity. The hybridization assay uses genomic and/or vector DNA, labeled randomly throughout the DNA, as the hybridization probe reagent. For this reason, the hybridization assay is specific for the source of DNA but is not specific for a given sequence. A synthesized probe, specific for a specific sequence, can be prepared and used in the hybridization assay if this level of specificity is desirable. The DNA-binding protein residual DNA

assay is not sequence specific and hence not specific for the host DNA. Therefore, laboratory personnel should avoid contaminating samples for this assay with environmental DNA before denaturing the DNA; otherwise, the DNA result may be falsely elevated. The qPCR probe has the advantage of being sequence specific, but this creates some special challenges for development of a qPCR residual DNA assay. The qPCR-specific sequence must be a stable sequence within a suitable region of DNA. The recovery of the target sequence must consistently represent the recovery of all the residual DNA. Biopharmaceutical manufacturing processes may typically include operations that shear DNA into smaller fragments, and this must be taken into consideration when selecting an assay. Procedures exist to determine whether the DNA fragments in a sample are too small for adequate residual DNA recovery with a given assay. When bridging from one DNA assay technique to another, a thorough understanding of the DNA analyte is critical. Some assays can detect both single-stranded and double-stranded DNA, whereas some can only detect double-stranded DNA (e.g., some fluorescent dye-binding assays). There are assays that are not sequence specific, and those assays that are sequence specific can be influenced by the copy number of the target sequence present in the DNA. There are assays that require two or more antibody molecules to bind to the DNA fragment (e.g., DNA-binding protein-based residual DNA assay), and if the DNA fragments are too small and present in sufficient quantity, they can saturate the reagents and inhibit the assay (hook effect).

Although safety concerns regarding residual DNA impurities are not as prominent as they once were, the levels of residual DNA in any bioprocess remain a key quality attribute and provides valuable characterization of the manufacturing process.