

When is my Oligonucleotide Pure Enough?

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This is a very common question we receive from our clients. How do you know what purity is needed and what to ask for? The answer is predicated on many factors and can be complicated. The decision will vary depending on whether it is a research scale or a commercial scale synthesis; if it is a novel compound or one we made several times already; how highly modified it is; what is its application; and so on. In the end, your TriLink Product Specialist is your best way to get the answers you need for your specific problem. However, there are several guidelines we can offer that will help you make some general decisions before you place a call for advice.

How is Purity Defined?

Usually, the percent purity seen on most Certificate of Analyses (COA) for an oligonucleotide is the result of the HPLC analysis of the final product. This number can be erroneous for many reasons. For example, contaminants probably exist that do not separate from the product peak. Reverse phase (RP-) HPLC can often be very poor at separating n-1, n-2 and even shorter fragments from the main product peak. Also, there are many other contaminants that can be in a product sample that do not absorb light at 260 nm, such as excess salts and purification matrix (especially in PAGE purified samples). It is very hard to determine the amount of salt in sub-milligram samples.

A sample that is 90% pure by RP-HPLC analysis observed at a wavelength of 260 nm may only be 80% pure by anion exchange (AX-) HPLC at the same wavelength. Furthermore, the same compound could be only 70% pure if free dye is measured at an alternative wavelength, or only 60% pure by mass if excess salt is taken into account. Generally, the analytical method chosen gives the purity based on the factors important to the application. Therefore, purity is defined by the method(s) of analysis, which is determined by the application.

Common Oligonucleotide Analytical Methods

Polyacrylamide Gel Electrophoresis (PAGE)

PAGE separates oligonucleotides on the basis of mass/charge ratios by passing an electrical charge across a gel matrix. The negatively charged oligonucleotides are drawn through the matrix. A common method of detection is UV backshadowing, where a UV lamp is shined on the gel which is on a fluorescent background. The oligonucleotides absorb the UV light, leaving a shadow on the background. Denaturing PAGE (the gel has a high urea content to force any double strands to separate into single strands during the analysis) is the most common analytical tool for the analysis of oligonucleotides.

PAGE is good for qualitatively examining a product. It is very effective for separating oligonucleotides based on length. However, it is a very poor quantitative tool and has a fairly narrow linear range. Better quantitation can be obtained if the oligonucleotides are radiolabeled prior to analysis, but this is costly and time consuming for commercial production.

PAGE analysis is inexpensive and very adequate for showing the basic quality of an oligonucleotide. In the case of basic primers and research compounds it is more than adequate as the sole final QC analysis for all but the most demanding applications. Every oligonucleotide sold at TriLink is analyzed by PAGE at no additional charge.

Spectral Analysis

Every oligonucleotide is quantitated by measuring the absorbance of a solution of the sample at 260 nm. Spectral analysis of oligonucleotides in both the UV and visible ranges

can also be very useful. We use the ratio of the absorbance of the oligonucleotide at 260 nm and the absorbance of a dye at its absorbance maximum to determine if any free dye remains in the sample. This is a very important assay and part of the final analysis performed by TriLink on every dye-labeled oligonucleotide as a free service.

High Performance Liquid Chromatography (HPLC)

Due to its versatility, HPLC has long been the most commonly used analytical tool for determining the purity of many classes of compounds. There are also many different classes of HPLC. The two most commonly used in oligonucleotide chemistry are reverse phase (RP), which separates on the basis of mass/lipophilicity ratio, and anion exchange (AX), which separates on the basis of anionic mass/charge ratio.

A RP matrix generally consists of a silica, glass or polymer particle coated covalently with a long chain hydrocarbon, the most common being octadecyl, or C-18. An AX matrix has a coating of cationic molecules, most commonly a form of diethylaminoethyl (DEAE) or a quarternary amine.

The most widely used and least expensive matrix available to oligonucleotide chemists historically was RP, and still is. AX chromatography was very difficult to perform in the early days because of manufacturing problems and reproducibility issues. On the other hand, RP matrices have over 50 years of commercial development behind them.

However, RP-HPLC has many problems, the most glaring being its inability to separate oligonucleotides of similar length, longer than approximately a 12mer (Figure 1). Sequence alone can greatly affect the retention of an oligonucleotide, to the extent that in a few cases a shorter fragment will retain longer than the full length product. For example, a product that contains a primary amino functionality for conjugation purposes will often elute along with the failure sequences because the amine is polar enough to change the lipophilicity of the oligonucleotide and overcome the mass difference.

AX-HPLC is very good at separating shorter fragments from the full length product (Figure 2, as compared to Figure 1). In more recent years it has become more reproducible.

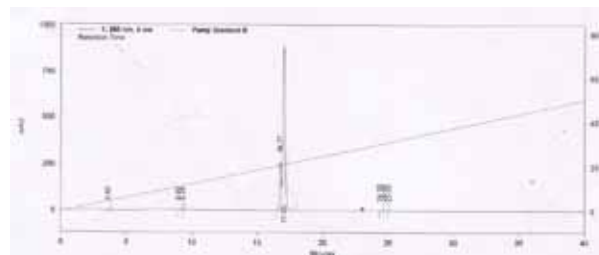


Figure 1: RP-HPLC analysis of a mixture of 6 polythymidine oligonucleotides: T12mer, T18mer - T22mer.

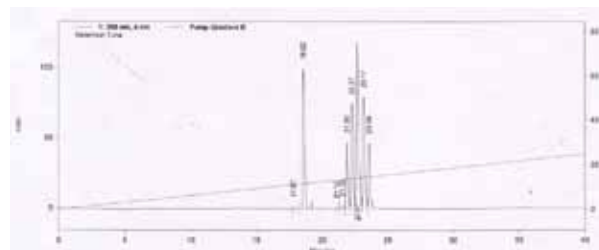


Figure 2: AX-HPLC analysis of a mixture of 6 polythymidine oligonucleotides: T12mer, T18mer - T22mer.

Analytical columns are much more expensive and do not separate some chemical modifications, such as dyes, as well as RP.

It should be kept in mind that it is difficult to predict with certainty the ability of any method to separate the contaminants of interest without synthesizing the suspected contaminants as analytical references. This is commonly done for the development of HPLC analytical methods for the analysis of pharmaceutical grade oligonucleotides. Therefore it must be understood that the purity number obtained from HPLC, especially when it is the only analytical method used, is only an approximation of the purity.

Which method is best, AX or RP-HPLC? That is difficult to answer definitively. It was already pointed out that the two methods are better at separating different types of contaminants. Having an analysis of your compound by both AX and RP-HPLC is better than either one alone; however, in general, analysis by AX is preferable. We often see more contaminants using this method. All syntheses of 50 mgs or greater are analyzed by AX-HPLC free of charge, as are many of our small scale modified compounds.

One significant problem that plagues HPLC is that it is not effective at separating fragments longer than 50-60 bases in length, and that is in the absolute best cases with AX. RP is much less effective for the separation of standard unmodified oligonucleotides, and without careful methods development must be considered unusable for anything over ten to twelve bases in length.

Mass Spectroscopy (MS)

Recently, this assay has become routinely available from oligonucleotide firms, including TriLink. MS analysis has become a great tool for the analysis of products and side-products. Determining the mass of a compound is an excellent way to help verify its identity and to determine unknown contaminants. However, this method also has limitations, some of them rather significant.

For instance, having a found mass identical to that calculated does not confirm a structure. For example, an oligonucleotide with the sequence ACTCGCTTGACAGAGT is a very innocuous looking 16mer that has the same mass as the self-annealing sequence CCCAAAATTTTGGGG, which has vastly different properties. In this case PAGE will easily differentiate between the two.

There are two MS methods typically used for the analysis of oligonucleotides, electrospray (ES) and MALDI. ES is very accurate, usually giving masses within 1 or 2 amu of the calculated figure. However, it is easily affected by the quality of the sample, particularly excess salts, necessitating stringent desalting and handling of the samples. MALDI is much more tolerant of sample quality, particularly salt content, but is easily thrown out of calibration. Further, the level of error can be large, hiding many modifications. In longer oligonucleotides the error can be as much as a whole nucleotide.

We use ES unless MALDI is requested, or in special circumstances where ES will not work.

Special Assays

There are many assays available to the oligonucleotide analytical chemist. In most cases their value is nowhere near their cost in time, money and material. Most are only needed for clinical samples or for oligonucleotides being used in special applications. In fact, the basic assays listed in the above sections are all that are needed for all GMP diagnostic and pre-clinical therapeutic syntheses, with perhaps the addition of an endotoxin assay in the latter case. Specifically, these assays include the aforementioned endotoxin assay, bioburden assay, capillary electrophoresis, base composition sequence analysis, hybrid melting temperature, water content, elemental analysis, NMR and metal content by atomic absorption. The costs of some of these assays are very high for what they return.

The endotoxin and bioburden assays are useful for late stage pre-clinical samples, particularly for material going into expensive mammals such as pigs, dogs or primates. The rest should only be considered if your regulatory department or the FDA requests them or if needed in your unique situation due to the composition of the compound, in which case most likely TriLink will suggest it in the course of developing specifications for the product.

An Application Driven Decision

Although it may be obvious that purity is determined by the application, the purity required by each application is not obvious. For instance, you may expect that a compound being injected into humans has a much higher purity requirement than a primer sold in a kit. That is not true in all cases. For instance, some assays require absolutely no contamination with particular sequences, especially target sequences, while a therapeutic drug can tolerate a small percentage of other sequences, as long as the individual components are positively identified and are reproducible from lot to lot.

Purity requirements also normally change as the product progresses toward commercialization. On the other hand, some assays require high quality material at the beginning just to allow development, with the introduction of robustness to the assay coming later in the process. This is often true in the development of very exacting microarray assays using dye labeled oligonucleotides. Since every situation is unique, the best we can do is offer basic guidelines for the following general classes of oligonucleotides ordered from TriLink.

1. General biological research grade, non-diagnostic primers:

Many of the compounds ordered world wide on a daily basis fall into this category. These consist of the oligonucleotides used for basic biological research, such as primers used for simple amplification or extension experiments. These compounds are often pure enough as crude desalts. In most cases a purity of 70% is more than sufficient. The impurities should be spread out fairly evenly among the possible shorter fragments. All oligonucleotides should be desalted to less than 20% salt by weight by either cartridge or precipitation at the least to remove excess salts that may interfere with enzymes.

Assays to request and results to expect:

PAGE is more than sufficient for these compounds. Make sure that the product bands are strong and that the contaminants are faint if observable at all. There should not be a large n-1 band; this is often indicative of other problems.

2. General biological research grade probes and modified oligonucleotides:

These compounds are generally only prepared once or twice at most with any particular sequence. Purity is often limited by the number and type of modifications. We attempt to achieve as close to 90% as possible, although sometimes we cannot achieve greater than 70% -80%.

For most applications in general research that use modified oligonucleotides, such as studying the physical properties of modified oligonucleotides or how they affect a biological system at a cellular level, 80% will often be more than adequate. Once again, we attempt to achieve at least 90% purity on every compound. However, there are some applications where higher purity may be necessary, usually in respect to a specific contaminant. For example, any free dye present in a labeled oligonucleotide sample can wreak havoc with most assays. Occasionally, there are some combinations of sequence, modifications and dye that do not allow for good purification.

Assays to request and results to expect:

In many cases PAGE is sufficient. If the material is purified, the band should be sharp and the contamination

very minimal. With some modifications and some sequences it is very hard to obtain products that are single bands on PAGE, however it should be very close to a single band, or an explanation should be accompanying the COA detailing why the data appears as it does.

For highly modified compounds, or for orders of 50 mg or more, a greater number of assays are used to ascertain the quality of the final compound. The suite still includes PAGE, since it does quickly show us problems that other methods do not. We also analyze the compound by AX-HPLC. We will include an analysis by RP-HPLC if needed. Both PAGE and HPLC analyses should show strong product bands or peaks with the contaminations each no more than a few percent of the overall mixture.

All dye labeled compounds are analyzed by UV/Visible spectroscopy to ensure that the dye still has the desired spectral qualities and did not undergo chemical degradation during synthesis. We also check that the oligonucleotide/dye ratio is correct for the number of dyes attached to the oligonucleotide. This is an approximation based on calculated extinction coefficients; however, repeat syntheses are compared to previous lots to ensure reproducibility. This data is hard to interpret and should be discussed with a TriLink representative if you intend to use this as a specification for future releases of a product.

Mass spectroscopy is also run on many small scale oligonucleotides and routinely on all 50 mg or larger orders free of charge. It is considered more of a qualitative, as opposed to a quantitative, method. It is also extremely valuable for process control, giving us an excellent tool to identify the appropriate product in a complex mixture.

3. Commercial diagnostic grade primers and probes:

The specifications for these products should be set no higher than what is needed for the assay to work routinely. The reason is that you want the assay to be robust, but at the lowest price possible. In this case, 80-90% is also usually good enough with the same careful attention given to the removal of free dyes or other interfering contaminants. Some specific applications may require higher purity, 95% or greater. This is actually quite rare. It must be understood that yield will be greatly affected as higher purity is desired. Some contaminants, such as free dye, must be removed as completely as possible.

Some sequences and/or modifications do not allow high purity. We are often limited by impure dyes in the manufacturing of fluorescent oligonucleotides.

Assays to request and results to expect:

All the assays discussed in the preceding section on general biological research grade products apply for this category of products. The only consideration here is that assays that may have been optional previously are now mandatory. Each relevant assay must be run. The desired results are set by the product specifications, which were in turn set by the validation runs, which establish the capabilities and reproducibility of the synthetic protocol. Of course, all specifications are subordinated to the product's required performance in the assay.

There are times when additional analysis is required. We do have the capabilities of testing the randomness of oligonucleotides bearing degenerate positions using a base composition assay. This special service requires enzymatic degradation of the oligonucleotide. Most of the other special assays are unnecessary for diagnostic products.

4. Therapeutic oligonucleotides:

The purity requirements for a therapeutic candidate are quite unique in many ways. This is one case where it is definitely not in your best interest to obtain as high a quality as possible at all times. In fact, we recommend a sort of concave profile for your product's purity as it runs its course through

research and into the clinic.

At the very early stages of research you want to be sure to be working with as high a quality of material as possible. Much time has been wasted as a result of poor quality syntheses leading to cytotoxicity. Therefore, you want a much higher purity than is normally available from most small scale providers. At TriLink we specialize in the synthesis of these types of compounds and understand how to reduce unwanted cytotoxicity.

Once a target molecule is prepared and is going to be scaled up for animal studies, a more complicated strategy must evolve regarding the purity of the product. The main consideration is that it is critical that the drug stays as pure, or better yet, gets increasingly purer during each stage of the development of the commercial manufacturing protocol. It is important to not set extremely high purity specifications when the drug is being prepared at a relatively small scale for use in pre-clinical animal studies and even early phase clinical trials. If that purity is not met when the compound is scaled up for later clinical phases, you may have to repeat all your earlier work with the newer material – a very costly mistake.

A good rule of thumb is to use 80-85% pure material for pre-clinical animal studies, 85-90% for Phase I toxicity studies and 90-95% for Phase II/III studies.

It is actually more critical to remove small molecule contaminants from the beginning of the program, such as organics and salts, than it is to have an extremely clean 260 nm profile on HPLC. We always use care to remove salts and other contaminants from oligonucleotides that are to be used in cellular or animal experiments. It is not possible to assay for some of the small molecule contaminants using standard assays, and the ones required are usually impractical during early stage research, but our expertise in this area is sufficient for you to trust our products for all early stage pharmaceutical research.

Assays to request and results to expect:

Again, all of the assays applicable described for use with research grade oligonucleotides will be used for these oligonucleotides. In your early research the normal high quality you have come to expect from TriLink will suffice for your research compounds. As your program progresses, the number and complexity of the assays automatically ratchet up as well. As you get closer to a Phase I clinical study your regulatory department will be exploring the specifications of the oligonucleotide and the assays required to file an IND. Assays that test the bioburden and endotoxin contamination become necessary for the batches made for the final pre-clinical higher order animal toxicity studies. Sequence analysis becomes critical, using either classical or newer MS methodology.

The more esoteric assays, such as metal content, etc., may or may not be required, depending on the viewpoint of your regulatory staff and their interactions with the FDA. In our experience, the FDA will not require much beyond that described above if the manufacturing protocols being followed are considered standard.

TriLink's alliance with Avecia, the leading manufacturer of oligonucleotide drugs for the clinic, is a wonderful resource for you at this stage. Avecia will take an active role in helping with assay development as soon as you are ready to discuss taking your program to the next level. Since our methods are harmonized, it will be easy for you to transition to clinical scale manufacturing.

Summary

Hopefully you found some useful hints in this article that will help you make decisions regarding the purity of your oligonucleotide. Please ask your TriLink representative for more guidance if needed. We are happy to help.