NORDIC BIOMARKER

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Design of D-dimer Applications

Introduction

For us, an instrument application is a number of settings in the software of a coagulation- or clinical-chemistry analyzer, to make the performance of a reagent as good as possible on that instrument. It could be any reagent, but in this guideline we will focus on D-dimer applications. We do this because on almost any coagulation analyzer, the D-dimer application is the most difficult and demanding, of all applications, to design. The routine coagulation reagents typically run on coagulation analyzers (e.g. PT, aPTT, Fib) are based on detection of clot formation; a large and dramatic event that in most cases is easily detected by the detection systems in these instruments. The chromogenic reagents (e.g. AT, Protein C) also have a large signal-to-noise ratio, making it relatively easy to design these applications.

When running latex immunoassays (LIA) on coagulation analyzers, however, the optical detection system of the instrument needs to observe a small change in signal, on top of a large background signal. This is especially true for D-dimer reagents, but the same basic problems are also encountered when running other latex-based reagents (e.g. vWF:Ag, Free Protein S) on coagulation- or clinical-chemistry analyzer. Therefore, it is extra important to systematically design and optimize the applications for LIAs. In this way, you will get the best performance possible from the system, and also probably save a lot of the time you would otherwise have spent on guesswork.

In the end, the goal we ultimately strive for is an application that is reporting correct and consistent D-dimer results to the end-user of the system. Therefore, before we put the reagents on the market, we would also need to do a more complete performance evaluation to verify and validate the application. That is, however, the topic of another guideline. In the current guideline, we focus mainly on designing the application that will later go through verification and validation. But, since verification and validation often are both costly and tedious projects, our application-design work becomes very important. Once we started verification and validation, we really don't want to re-design the application. Therefore, in the current guideline, we will also give you some suggestion on what to include in a small preliminary verification of the application.

Fundamentals of Latex Immunoassays

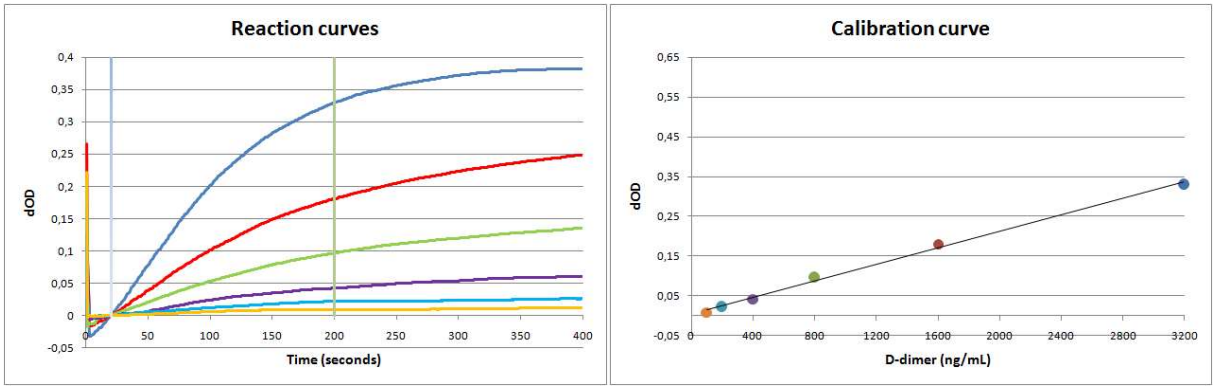
D-dimer reagents are latex immunoassays (LIA) which rely on turbidimetry for their function. This means that the reaction is detected by observing the change in turbidity, or light-scattering. The latex

particles are coated with monoclonal antibodies specific for D-dimer[1], which, when suspended in water, will look like milk. The particles scatter light, which make them look white and milky (incidentally, this is also the same reason why milk looks like milk!).

Before starting the reaction of a LIA, patient plasma sample and reaction buffer is first added to the reaction chamber of the instrument, the cuvette. After some incubation time, to allow for the temperature of the mixture in the cuvette to reach the desired value, latex reagent is added to start the reaction. Then, the D-dimer that was present in the patient sample, will start to bind to the monoclonal antibodies attached to the latex particles. Since D-dimer is a dimer, and as such has two binding sites for the antibody, another latex bead can bind to the same D-dimer. Since each bead has many antibodies on its surface, each bead can bind many D-dimes. Therefore, as the reaction progresses, the latex particles will go from being mono-spheres, to being bi-spheres, tri-spheres, and so on. The larger species will scatter more light, which is why the turbidity will increase. This whole process takes only a few minutes, while the change in turbidity is being monitored by the instrument's optical system. The rate of this change is proportional to the amount of D-dimer antigen in the sample, allowing quantitative determination of D-dimer.

The change in turbidity is usually monitored as the change in absorbance, or optical density, (OD) over time. Figure 1 (left panel) shows six such reaction curves, for a calibration series with falling D-dimer concentrations. As can be seen by observing the upper reaction curve (3200 ng/mL in blue), the absorbance increase over time is non-linear. In fact, none of the reaction curves are perfectly linear, but the non-linearity is mostly pronounces for the reaction curve with the highest sample concentration. This non-linearity has to do with the complex changes in physical and optical properties of the aggregates that occur as they grow bigger. We need to keep this non-linearity in mind as we go along.

Figure 1: (Left panel) typical reaction curves of D-dimer latex, at six different D-dimer concentrations falling from 3200 to 100 ng/mL. For ease of observation, the OD at 20 seconds was set to zero. (Right panel) Example calibration curve constructed from the six reaction curves by plotting dOD between 20 and 200 seconds.



[1] D-dimer refers to a mixture of crosslinked fibrin degradation products (FDPs) that each contain two or more D-D domain motifs.

The right panel in Figure 1 shows the simplest way to extract a calibration curve from a set of reaction curves; the change in absorbance in a certain time frame (dOD) is plotted against concentration. Later on, we will show that there is more information to extract from the reaction curves.

Choose the right D-dimer kit for your instrument

Nordic Biomarker has developed three different D-dimer reagents:

- Red D-dimer suitable for instruments with optical channels in the range 650 – 800 nm. That is, most larger coagulation analyzer as well as practical all biochemistry analyzers can run Red D-dimer.
- Green D-dimer suitable for instruments with optical channels in the range 500 – 650 nm. STA-instruments and several STA-clones operate at these wavelengths.
- Blue D-dimer suitable for instruments operating at 405 nm, a common wavelength in all coagulation analyzers, and the only choice in many smaller instruments.

The main difference between these three D-dimer reagents is the size of latex particles used to produce them. Of course, all three LIAs appear white, when looking at them in real life. The names Red, Green and Blue is just a mnemonic trick to remember which wavelength the are best designed for: red light has long wavelength, blue has short, and green is in the middle!

The light-scattering properties of latex particles depend mainly on two variables: the size of the particles and the wavelength of the light. Small particles would not work well at long wavelengths, whereas a large particle would behave poorly at shorter wavelengths. Therefore, Nordic Biomarker has developed the three different D-dimer reagents to cover most coagulation- and biochemistry analyzers available on the market today.

Almost all analyzers on the market have the wavelength 405 nm as an option. Larger analyzers have several other optical channels to choose from too, but they usually also have 405 nm. Smaller semi-automatic coagulation analyzers may have 405 nm as the only option. For this reason, Nordic Blue D-dimer is designed to work well at 405 nm.

If almost all analyzers have 405 nm as an option, why not just run Blue D-dimer on all of them? Why, then, develop at all other D-dimer products, such as Green and Red D-dimer? The answer is interfering substances. To get enough D-dimer in the cuvette for the latex immunoassays to function properly, a relatively large amount of plasma sample is needed. Along with the D-dimer in the plasma sample, there are also some interfering substances like, for instance, hemoglobin, billirubin and lipids. At low wavelengths, such as 405 nm, the absorbance from these interfering substances is very high. Sometimes high enough to cause instrumental errors, which will result in extra testing and investigations for such plasma samples. At higher wavelengths, however, these interfering substances are usually not a problem. Therefore, if you have an instrument in your laboratory that is capable of running at higher wavelengths you should definitely choose Green D-dimer, or, even better, Red D-dimer.

To choose the right kit for the instrument in question, and to do a few important calculations downstream in the application process, here are the three questions you must first have answers to:

- Which wavelength(s) can the instrument use for latex immunoassays?
- What is the optical path length of the cuvette?
- Which is the minimal working volume of the cuvette?

With the answers to these questions at hand, we can start to build an application in a theoretical way, before we even go into the laboratory to test it. If we do this right, we might save a lot of time further down the line.

Determine the amount of Latex Regent in the cuvette

So, as mentioned above, the reaction mixture is milky and turbid even before the reaction starts. As turbidimetry relies on absorbance, and as the instruments used really are spectrophotometers, we have to consider Lambert-Beers Law. Briefly, this means that the absorbance must lie well below 2.0; above 2.0 the light that reach the detector is too weak for a good signal-to-noise ratio. In addition, as the absorbance increases during a measurement, the initial absorbance must be even lower. A good starting point is typically in the region of 1.0 absorbance units.

As many coagulation instruments do not display the true absorbance value on screen, a practical way to obtain a rough estimate is to simply dilute the latex reagent in the D-dimer kit tenfold with water, and measure the absorbance, using a standard spectrophotometer, at the same wavelength as you will later use on the coagulation instrument. For instance, if you do this with the Latex Reagent in Nordic Blue D-dimer, you will get an absorbance reading of 0.41 in a 1 cm cuvette at 405 nm. Hence, the absorbance value of the undiluted latex is 4.1. Say, for instance, that the optical path length of your coagulation instrument is 0.6 cm, then you would need a (4.1/1.0)*0.6 = 2.5 dilution of the latex reagent in the cuvette to reach a start absorbance of 1.0.

Further, if the minimal volume of the cuvette is e.g. 150 µL, the volume Latex Reagent that should be used is 150 µL / 2.5 = 60 µL.

In the real application, the Latex Reagent should be added last into the cuvette to start the reaction. As good mixing is important to get a homogenous suspension of particles, the instrument must be well designed to achieve this. However, if the volume of Latex Reagent is too small, no instrument in the world will be able to achieve a good mixing. Therefore, as a rule of thumb, the volume of latex should not be less than 1/3 of the total reaction volume. If it is, you need to review other options (different wavelength and/or different reagent).

The volume of Latex Reagent should not be too large either. That is because room must be left also for the Reaction Buffer. The Reaction Buffer is important to provide the right milieu for the reaction. Therefore, you should try not to exceed a volume of Latex reagent that is more than 2/3 of the total reaction volume.

Determine the amount of sample in the cuvette

The amount of sample in the cuvette is a compromise between, on the one hand, precision and accuracy in the lower region, and, on the other hand, the upper limit of the measuring range. In other words, with a large sample volume in the cuvette, the absorbance change during the measurement will be large even for samples with low concentration of D-dimer, giving good precision. The drawback with a large sample volume, however, is that it will produce non-linear reaction curves at lower D-dimer concentrations than if the sample volume had been low; hence reducing the upper reportable range. Conversely, using a small sample volume in the cuvette will give linear reaction curves even at higher D-dimer concentrations, but very small dODs with poorer precision at low D-dimer concentrations.

Fel! Hittar inte referenskälla. showed an example of a rather balanced situation. The reaction curve for 3200 ng/mL is linear up to about 100 seconds, and a sample with 100 ng/mL D-dimer still produces a decent signal-to-noise (although it is difficult to appreciate this in the scale of the figure).

Generally, a 1/10 volume of sample is good starting point for a D-dimer application. We can then adjust it up or down later based on the results. We will come back to this topic later. It is certainly worth discussing more, and it is perhaps the best way to tweak the application in a more favorable direction. But if we start with sample volume that is 1/10 of the minimal working volume of the cuvette of, say, 150 µL, the sample volume we should start with would be 15 µL.

Determine the amount of Reaction Buffer in the cuvette

This will simply be the volume of Latex Reagent and sample subtracted from the cuvette volume. In our current example: 150 µL – 60 µL - 15 µL = 75 µL.

Try the application on the instrument

By reasoning we have now arrived at the starting conditions for our application. We have first decided which D-dimer kit to use, and then calculated the amount of the components. The example application here is,

15 µL sample
75 µL Reaction Buffer
65 µL Latex Reagent

One minute incubation at 37 °C, before the Latex Reagent is added, is usually enough. The Latex Reagent should then be added last to start the reaction. A good mix of the entire cuvette contents, when the Latex Reagent is being added, is important.

It is now time to program the application into the instrument. If possible, we also want to program the instrument to serial dilute the D-dimer Calibrator, and execute the application for each dilution. Even if it may be too early, the instrument software may require us to enter a start and stop time for the calculation of dOD; if so we should just guess something generic like 20 and 200 seconds, respectively. The total reading time, however, is more important at this stage. That is because we do not want to miss seeing the reaction curves for a long time during this initial phase. Later, when we have decided the final stop time, there is no reason to keep the instrument busy by collecting extra data, but for now we suggest 400 second for the total reading time.

Without seeing the actual reaction curves, i.e. how the absorbance changes over time, we are really fumbling in the dark. On some older instruments, however, it is not possible to actually see the reaction curves; in this case it will merely report a dOD for a certain time frame. For these older instruments, which lack modern software where we can easily view reaction curves, the iterative process that will follow can be considerably longer.

When you have successfully programmed the application into the instrument, added all reagents, calibrator and Dilution Buffer into their appropriate positions in the instrument, you should run the calibration series. The reaction curves may look like the ones in Figure 1 (left panel). If there is poor separation between the slowest reaction curves (corresponding to the lowest dilutions in the calibration series), and/or the fastest reaction curve is more or less linear, you probably need more sample in the cuvette. Conversely, if the fastest reaction curve is hooked, and you already have good signal separation in the lower region, you can probably decrease the sample volume.

Predicting the necessary sample volume is a complex issue which depends on optics, wavelength, and reagent, and cannot be done perfectly in advance. The only way is testing a few different volumes on the actual instrument, and observe how it affects the reaction curves.

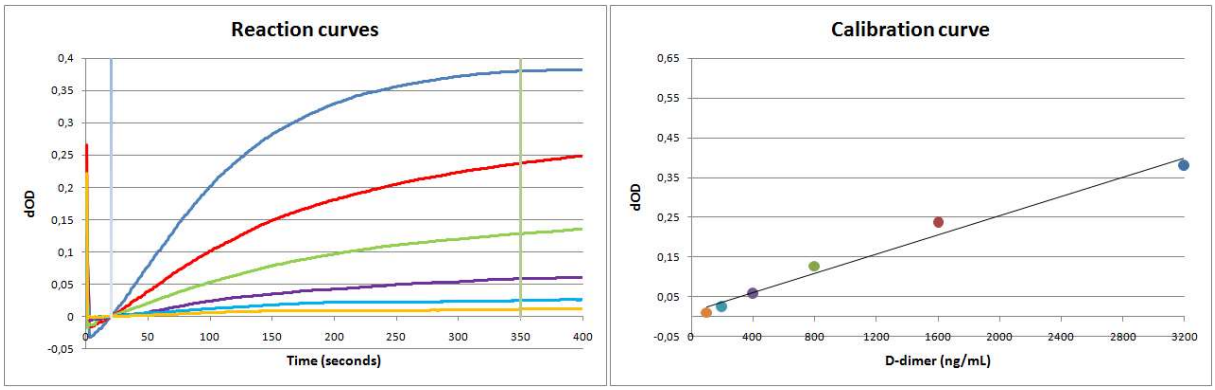
Decide the time frame for data collection

The reaction curves in Figure 1, is a good example of how to think when deciding how the instrument should interpret the data, to get the most out of the reaction curves. The following examples shows you how to construct a normal calibration curve based on dOD against D-dimer concentration.

Depending on how the instrument mixes the sample, the first few seconds will be more or less noisy. This data should not be included in the reading frame for data processing. By observation of the actual reaction curves that comes from the instrument, a start time for data processing should be chosen

that excludes the initial noise. On the other hand, setting the start time too high, will exclude data that is really needed for a good calibration curve. A start time around 10 or 20 seconds is normal.

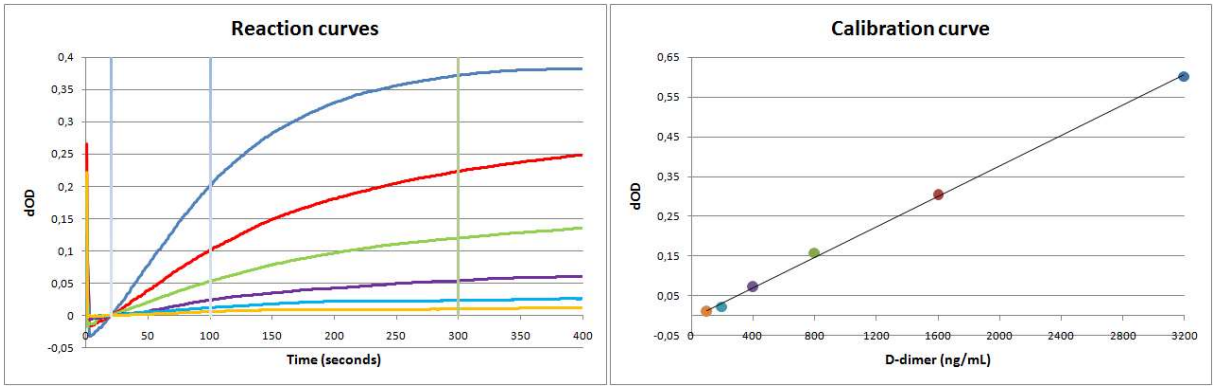
Deciding the stop time for the dOD, is a bit more difficult. It is often a compromise between calibration curve linearity and lower detection limit. For example, the application/calibration curve in Figure 1 could possible give rise to poor precision in the lower region, as dOD for low D-dimer samples could be too low. Therefore, it would be tempting to increase the stop time. Figure 2 shows this situation for the same reaction curves.



The signal/dOD in the lower region has indeed increased, but it is more or less un-changed in the upper region, giving rise to a non-linear calibration curve. Is this bad? No, not necessarily, provided that the instrument is capable of fitting a non-linear calibration curve to the data, or point-to-point. In fact, buying better precision in the lower region, and trading off a bit of the linearity in the upper region, is often preferable as long as it is not taken to extremes. Even if the slope of the calibration curve is lower in the upper measuring range, it is still positive and probably good enough (i.e. even if the accuracy is poorer than in the linear case, usually the clinician would not bother if the assay results is 2300 or 2500; it is still way above the clinical cut-off!)

In more extreme cases, where the calibration curve has more or less plateaued in the upper region, the test will suffer from poor performance with a prozone effect already at low D-dimer concentrations.

Many newer instruments are capable of using two different algorithms for dOD calculations, or even a dynamical function with multiple algorithms. The two algorithms are simply a way to use a long reaction time for dOD calculation for low D-dimer samples, where it is needed, and a short reaction time for high D-dimer samples. The second algorithm uses the slope to extrapolate dOD outside the reading frame, or will normalize slope against time (e.g. dOD/min). Figure 3 gives you an example.



With one algorithm for the lower region, and another for the upper region, good precision and linearity can be achieved at the same time. That is, getting more out of the data.

Final words

With good starting conditions, by looking at the reaction curves, and by changing the settings in an iterative fashion to achieve better results, the application can be optimized for better output. When you have reached that point, it is important to look over other aspects of the application, such like e.g. re-run rules for very high samples. To the extent that it is possible on the instrument, it is important to set high-dose warnings at well chosen levels, to filter out very high samples. Some instrument uses the initial slope of the reaction curve for high-dose warnings, whereas others use non-linearity thresholds of the reaction curve as high-dose warnings.

After this, the application needs to be verified. We suggest that you first check the CV in the lower end, and prozone effect in the upper end, to roughly find your lower and upper measuring range, before you embark on the full verification. If the CV in the lower end id great, but the linearity poor in the upper end, reducing the sample volume will probably do the trick.

Last, a final word of warning. Some coagulation analyzers, and specifically their cuvettes, were not at all designed for LIAs. Measuring turbidimetry poses some additional problems other than measuring just the absorbance change at clotting or for chromogenic assays. Unfortunately, some instrument manufacturers are still unaware of the extra requirements for LIAs, and only test clotting assays during the development of their instruments. Only to later realize that LIAs perform rather poorly on these instruments, when it is too late to change the design of the cuvette. This was perhaps not such a big problem 15 years ago when D-dimer assays were still rather unusual in many coagulation laboratories. Quite different from today when D-dimer assays are essential to almost all coagulation laboratories, and other LIAs for testing e.g. Free Protein S and vWF:Ag, are becoming more and more usual. So, the take home message is: if you are an instrument manufacturer developing a new instrument, test D-dimer assays early on! If you are working for a hospital laboratory and plan to buy new instruments for your laboratory, test D-dimer assays before you buy and look at that cuvette!

Nordic Biomarker is happy to provide further help and assistance if needed!