

# Design of D-dimer Applications

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Delivering a kit with vials is only part of the product. Just like a poor instrument application can make any reagent look bad, a good application can get the best out of the system. We have extensive experience in the design of instrument applications. As our customer, Nordic Biomarker will help you make the reagents perform its best on your instrument.

## Introduction

An instrument application is a number of predefined settings in an instrument. The application is designed with a particular reagent in mind, to give the reagent optimal performance on that instrument. As the title of this document indicates, it is focused on how to design D-dimer applications. The principles of how to do this are valid also for many other reagents. However, we found it easier to be specific and write the text with only D-dimer reagents in mind.

Nordic Biomarker's D-dimer kits are micro-particle enhanced immunoassays (often referred to as "latex assays") which rely on turbidimetry for their function. When the Latex Reagent, having monoclonal antibodies against D-dimer attached to the particles' surface, is mixed with patient sample and Reaction Buffer, the particles will agglutinate and give rise to an increase in light scattering. When this reaction is monitored in an instrument, having the necessary photometric features, it will be observed as an increase in absorbance over time. The rate of this reaction is proportional to the amount of D-dimer antigen in the sample, allowing quantitative determination of D-dimer.

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, a full validation of the application is needed. However, it would be unrealistic to do a full blown validation of each small application change that usually takes place during the iterative process that encompasses the design phase of the application. Instead we try to achieve the goal partly by common sense and reasoning, and partly by monitoring a few key performance indicators. With the current document, we aim to help anyone, who wishes to design their own D-dimer application, to do so in a systematic manner.

Then, in the end, when the design of the application is ready, we do need to validate the final application. Please find our recommendations about validation in another document: *Validation of D-dimer Applications*.

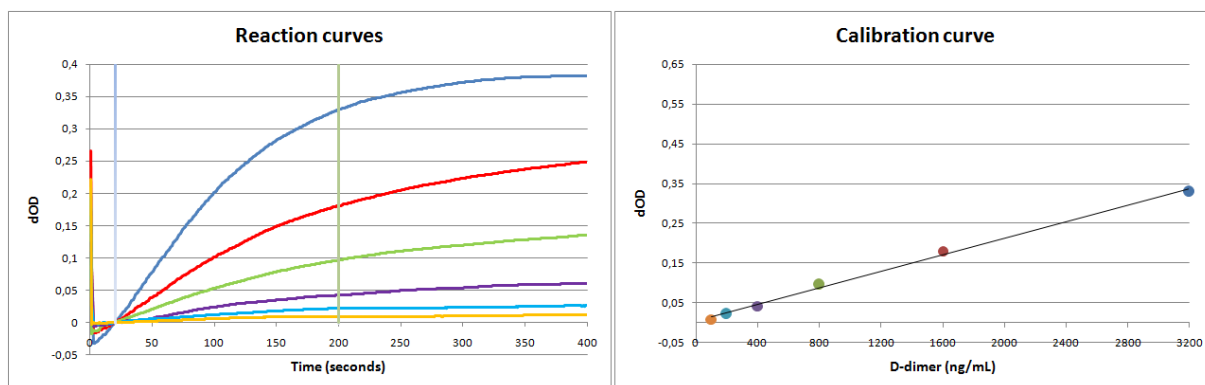
## Fundamentals of Latex Immunoassays

A Latex Immunoassay (LIA) is typically started by adding the latex reagent into a cuvette, and mixing it with the sample and reaction buffer that was already in the cuvette. If you don't already have it, you need an understanding of what is really going on in the cuvette of the instrument, when the reaction that follows takes place.

By simply looking at a glass vial containing latex reagent, it is evident that the particles scatter light; the liquid is “turbid”. So, when we add the latex reagent to the cuvette we already have a turbid liquid, before any reaction has taken place. Then, D-dimer will start to bind to the monoclonal antibodies that the latex beads have been coated with. As D-dimer is a dimer, and has two<sup>1</sup> binding sites for the antibody, another latex bead can bind to the same dimer. Since each bead has many antibodies on its surface, each bead can bind many D-dimers. Therefore, as the reaction progresses, the beads will go from being mono-spheres, to being bi-spheres, to tri-spheres, and so on. The latter species scatter light more, which is why the turbidity will increase.

What we aim to observe, and that is the basis for the entire assay, is how much, or how fast, the turbidity increases over time, as the reaction progresses. This change in turbidity over time is usually monitored by the change in absorbance (or optical density, OD). 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 pronounced 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.**



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.

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

## Choose the right D-dimer kit

As some instruments have optical channels only at lower wavelengths (e.g. 405 nm), whereas others only are capable of measuring only at higher wavelengths (e.g. 700 nm), Nordic Biomarker has developed two different D-dimer kits to cover a wider spectrum. The particle size in Nordic Red D-dimer (Part # K5002) is suitable for instruments with optical channels in the range 600 – 800 nm. At higher wavelengths, the light scattering properties of the particles decrease, which will lower the signal. Conversely, at lower wavelengths the particles would scatter too much light, reducing the residual signal seen by the detector close to its noise level.

In an analogous manner, the particles size in Nordic Blue D-dimer (Part # K5002) is chosen for it to be suitable for instruments with optical channels in the range 400 – 600 nm. However, there is really no magical cut-off wavelength below which Red D-dimer suddenly will not work, or above which Blue D-dimer will not work; in reality it is a compromise between cost and performance. For instance, Blue D-dimer could theoretically work at 700 nm, but since its light-scattering properties are poor at this wavelength, the amount of Blue D-dimer Latex Reagent needed in the cuvette would be high and hence also the cost. Conversely, if Red D-dimer were used at 405 nm, only a slight amount of Red D-dimer Latex Reagent could be added to the cuvette, to keep the light-scattering at a reasonable level, with poor performance (read, few antibodies in the reaction mixture) as a result.

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?

Even with the answers to these questions it may not be crystal clear which kit to choose. Although the wavelength is the major rule of thumb in this decision, the optical path length and minimal cuvette volume may also influence the choice; at least in the intermediate wavelengths around 600 nm.

In the event you have an instrument that is technically able to run either Nordic Blue D-dimer at a low wavelength, or Nordic Red D-dimer at a higher wavelength, the latter kit should be used. This is due to commonly interfering substances like hemoglobin and bilirubin. The absorbances of the substances are much, much higher at low wavelengths. Therefore, a high wavelength is a safer choice to reduce the interferences. Also lipids interfere less at high wavelengths, although the difference is not profound.

## Determine the amount of Latex Reagent in the cuvette

So, as stated above, there reaction mixture is turbid already at the onset of the reaction. As turbidimetry relies on absorbance, and as the instruments used really are spectrophotometers, one has to consider Lambert-Beers Law. Briefly, this means that the absorbance must lie beneath 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 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  $\mu\text{L}$ , the volume Latex Reagent that should be used is  $150 \mu\text{L} / 2.5 = 60 \mu\text{L}$ .

In the real application, the Latex Reagent should be added last to the cuvette to start the reaction. As good mixing is important to get a homogenous suspension of particles at this stage, 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 do a good mix. 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 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

Generally a 1/10 volume of sample is good starting point. However, the amount of sample in the cuvette is a compromise between, on one side, precision and accuracy in the lower region, and, on the other side, 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 here. The drawback with a large sample volume, however, is that it will produce non-linear reaction curves for the high D-dimer concentrations, hence reducing the upper reportable range. Conversely, using a small sample volume in the cuvette will render linear reaction curves even for high D-dimer samples, but very small dODs with poor precision for low D-dimer samples.

**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).

We will come back to sample volume later. It is certainly worth discussion more, and it is perhaps the best way later on to tweak the application in a more favorable direction. Thus, a 1/10 volume is just a good starting point. We shall be open for a change of this volume later on, depending on how the reaction curves looks. Anyway, to continue our imaginary example with a cuvette having a minimal volume of 150  $\mu\text{L}$ , the sample volume we would start with would be 15  $\mu\text{L}$ .

### **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 example  $150 \mu\text{L} - 60 \mu\text{L} - 15 \mu\text{L} = 75 \mu\text{L}$ .

### **Try the application on the instrument**

By sound reasoning we have now arrived at good 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  $\mu\text{L}$  sample  
75  $\mu\text{L}$  Reaction Buffer  
65  $\mu\text{L}$  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. If possible, we also want it 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 now we suggest 400 second for the total reading time.

Without seeing the actual reaction curves, i.e. how the absorbance changes over time, you are really fumbling in the dark. On some older instruments, 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, and added all reagents, calibrator and Dilution Buffer into their appropriate positions in the instrument, run the calibration series.

The reaction curves may look like the ones in Figure 1 (left panel). If there is poor separation between the two slowest reaction curves (corresponding to the two 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.

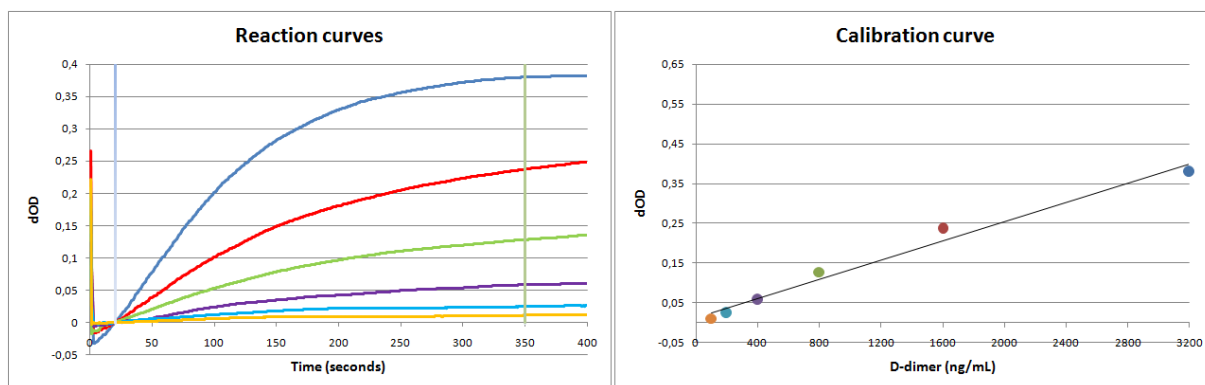
Predicting the necessary sample volume is a complex issued depending on optics, wavelength, and reagent, and cannot be done perfectly in advance. The only way is testing a few different volumes on the actual instrument, and observe how it affects the reaction curves.

### Decide the time frame for data collection

The reaction curves in Figure 1, is a good example on 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 from 10 to 20 seconds is normal.

Deciding the stop time for the dOD, is a bit more tricky. 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.

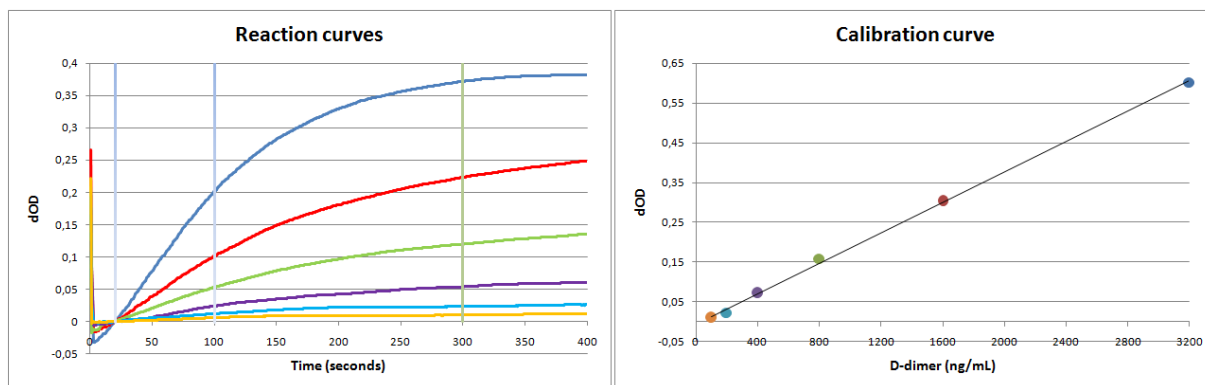


The signal/dOD in the lower region has indeed increase, but is more or less un-changed in the upper region, giving rise to a non-linear calibration curve. Is this bad? No, not necessarily, provided that the instrument is capable of fitting a non-linear calibration curve to the data, or point-to-point. 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 (see *Validation of D-dimer applications*).

Many newer instruments are capable of using two different algorithms for dOD calculations, or even a dynamical function with multiple algorithms. The two algorithms is 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 best 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 (See *Validation of D-dimer Applications* for an explanation of the prozone effect). 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.

This text was for you who want to do the application works on your own, and have not done it before. However, if you run into trouble, or have comments on this text, please contact us at Nordic Biomarker. We'd like to help you. That is why we are here, in the first place!