



## Enzymatic Chromogenic Assays LMW-Heparin

Author: B. Fehrmann (LEO Pharma)

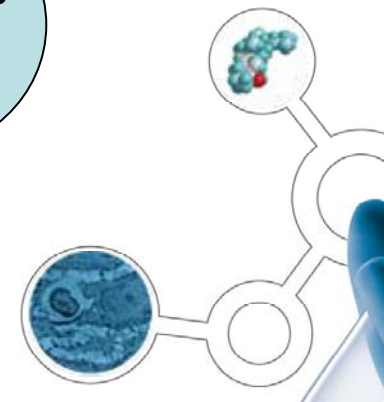
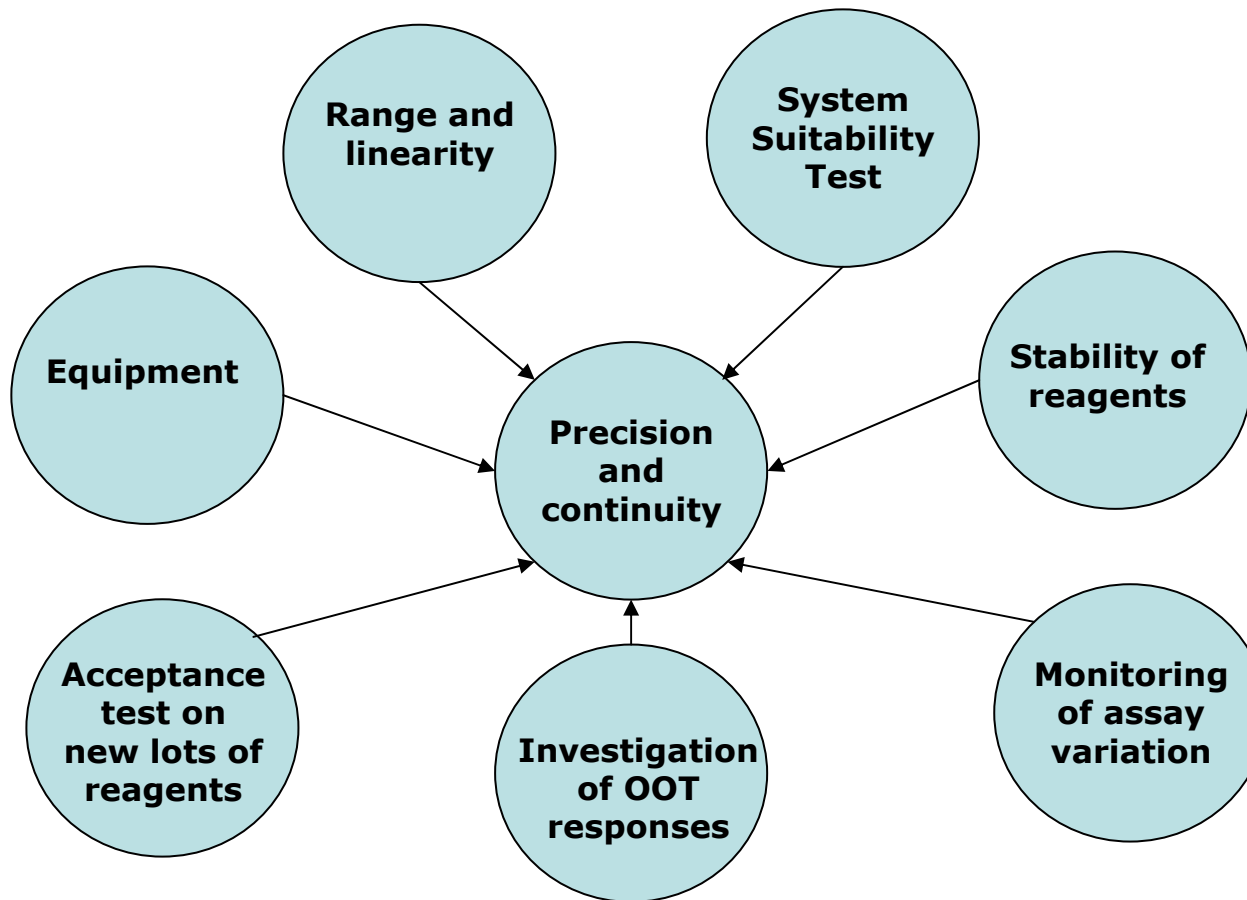
Presenter: R. Lecky (LEO Pharma)

**Achieving and Maintaining Reproducibility  
and Continuity in Anti-factor Xa and Anti-  
factor IIa Assays**



## Enzymatic Chromogenic Assays – LMW-Heparin Achieving and Maintaining Reproducibility and Continuity

- Schematic overview of the most important components in securing a stable and reliable enzymatic chromogenic assay





# Enzymatic Chromogenic Assays – LMW-Heparin Achieving and Maintaining Reproducibility and Continuity

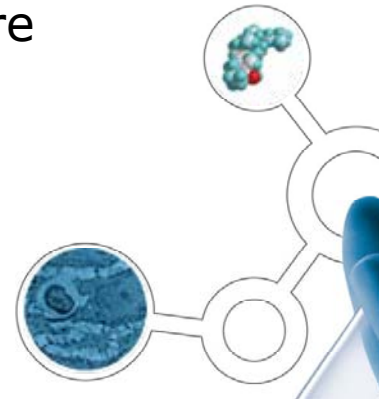
## Equipment – Choice of Equipment

The precision of enzymatic chromogenic assays is highly affected by:

- Variation in pipetted volumes of samples/reagents
- Variation in the timing of incubations
- A non uniform temperature distribution in the incubation area (can be a challenge when using analyzers equipped with a plate reader incubator with no or insufficient circulation of air)

Therefore:

- 1) The assay operations should be as automated as possible
- 2) Choose an enzymatic analyzer with a uniform temperature distribution in the incubation area





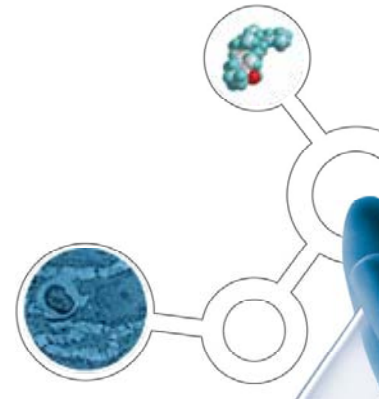
## Enzymatic Chromogenic Assays – LMW-Heparin Achieving and Maintaining Reproducibility and Continuity

1) The assay operations should be as automated as possible:

Choose a fully automated enzymatic analyzer that comply with the following:

The RSD% of 15 absorbance readings of 15 independent pipetted volumes of a potassium dichromate solution:

$RSD\% \leq 1.0\%$



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## 2) Choose an enzymatic analyzer with a uniform temperature distribution in the incubation area

The problem with some plate reader incubators is that the air inside the incubator is not circulated. This results in a non uniform temperature distribution e.g. the cells closest to the heater have a higher temperature than the rest of the cells.

Fig. 1 shows the poor temperature distribution of a plate reader incubator we once tested.

Highest temp.: 37.2 °C

Lowest temp.: 36.5 °C

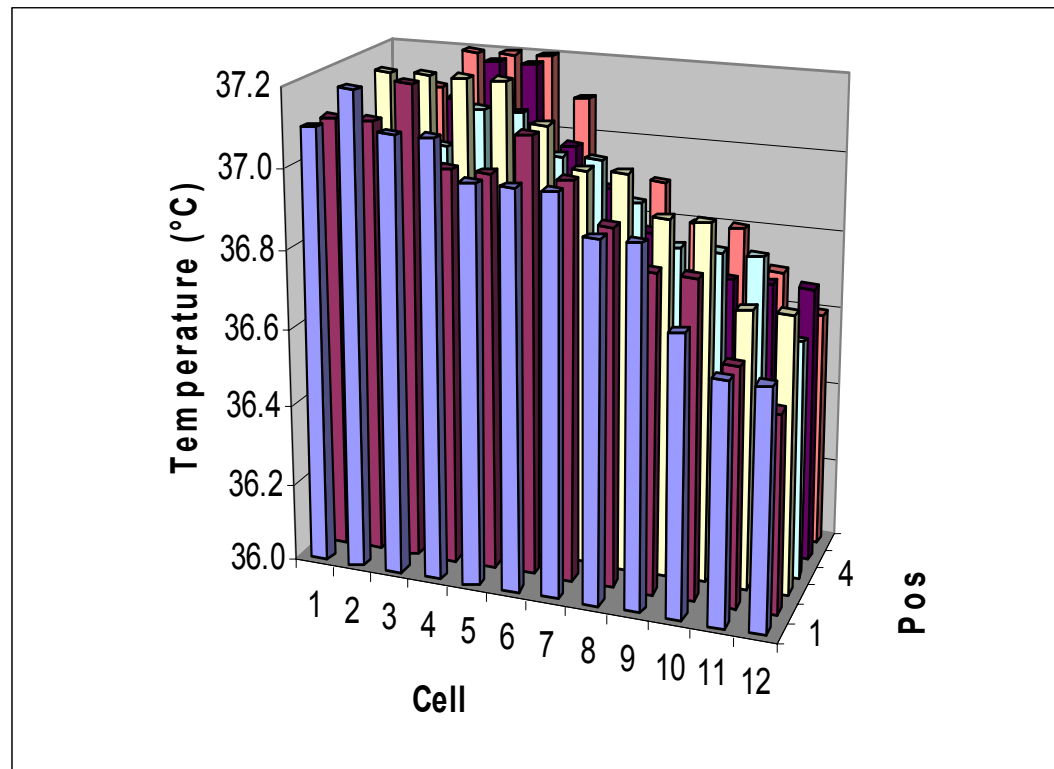
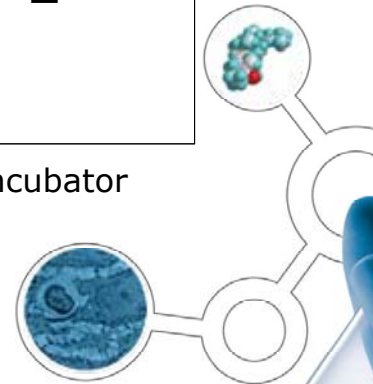


Fig. 1: Temperature distribution of a plate reader incubator



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The poor temperature distribution resulted in a correspondingly poor uniformity in the absorbance readings across the plate.

We rejected the analyzer due to these results.

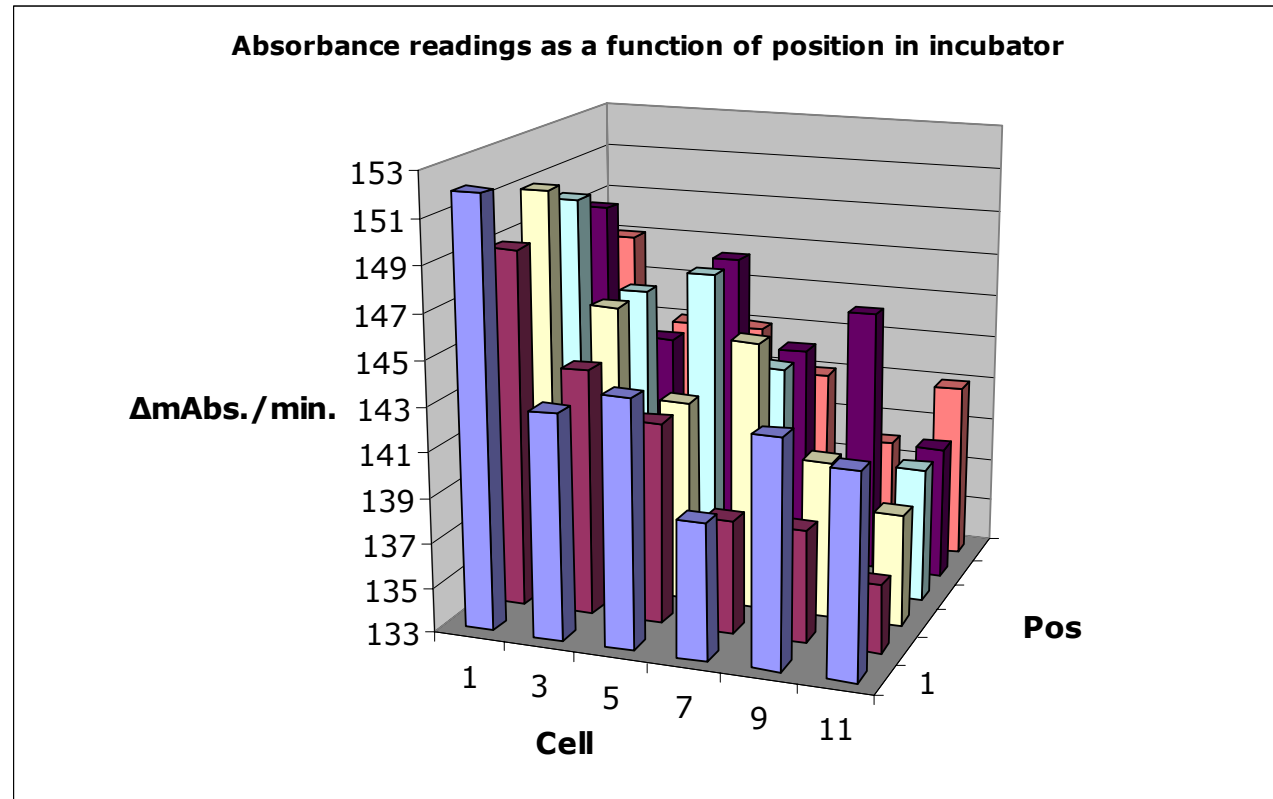


Fig.2: The same sample of tinzaparin sodium was analyzed using our Anti-factor Xa assay procedure. Only every second row of reaction cells across the plate were used as the other cells were filled with pH 8.4 buffer in order to improve heat distribution.





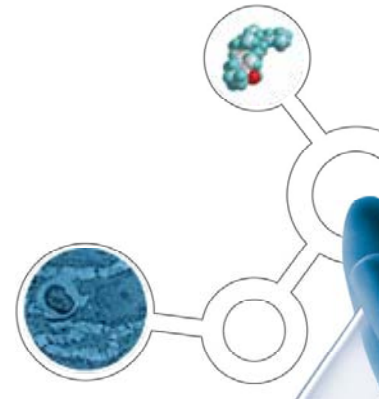
## Enzymatic Chromogenic Assays – LMW-Heparin Achieving and Maintaining Reproducibility and Continuity

Therefore choose an enzymatic analyzer with a good temperature distribution in the incubator, e.g. a build-in incubator with air circulation.

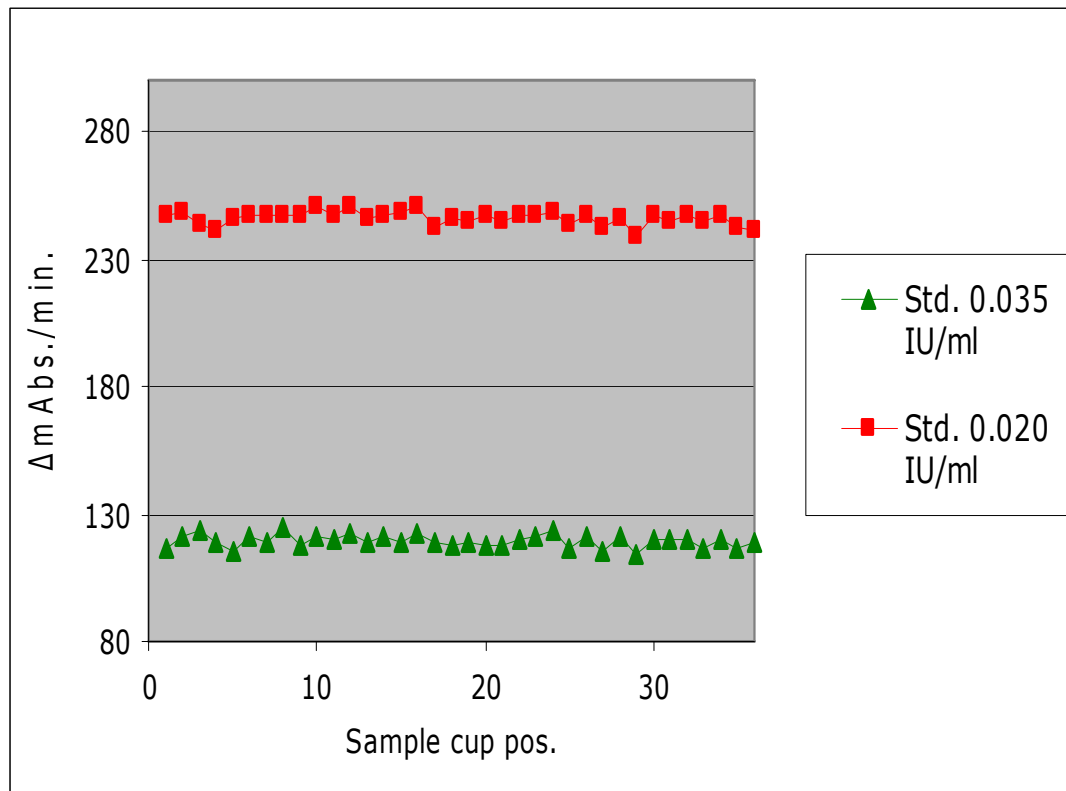
An example of a specification for a build-in incubator with air circulation:

Temperature in all positions: 36.8 °C to 37.2 °C

With this kind of uniformity in the temperature distribution you can obtain a fine repeatability through out all the positions of the incubator (see Fig. 3 on the next slide)



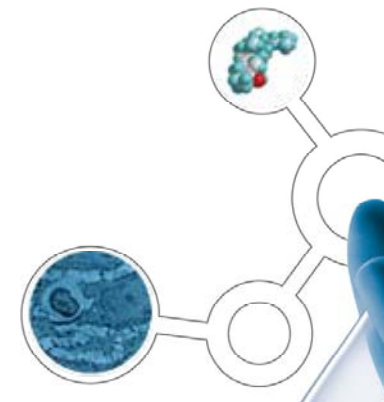
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Std.	0.035 IU/ml	0.020 IU/ml
SD ( $\Delta m \text{ Abs./min.}$ )	2.5	2.6
RSD%	2.1	1.1
Mean ( $\Delta m \text{ Abs./min.}$ )	119	246
Min ( $\Delta m \text{ Abs./min.}$ )	114	239
Max ( $\Delta m \text{ Abs./min.}$ )	124	251

Table 1: Descriptive statistics from repeatability study

Fig. 3: Anti-factor IIa assay repeatability using an enzymatic analyzer with a build-in incubator and air circulation (72 place incubator). Std. 0.035 IU/ml = Solution of tinzaparin sodium containing 0.035 IU/ml of Anti-factor IIa activity. Std. 0.020 IU/ml = Solution of tinzaparin sodium containing 0.035 IU/ml of Anti-factor IIa activity





## Enzymatic Chromogenic Assays – LMW-Heparin Achieving and Maintaining Reproducibility and Continuity

### Equipment – Calibration and Maintenance

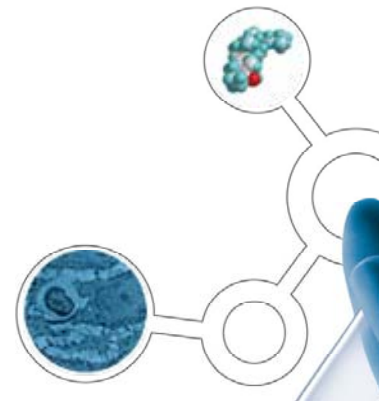
Use your normal assay method principles to develop an in-house test for calibration of the enzymatic analyzer, e.g. perform 10 replicate determinations of the highest concentration of your reference substance (RS) on the analytical level.

Set-up limits for the RSD% and the absorbance level in average ( $\Delta$ Abs./min.), e.g.

$RSD\% \leq 3.0\%$

$\Delta$ Abs./min. should be at the normal level for your assay. The exact limits differ from assay to assay.

(normal level = the average of all absorbance readings e.g. from one year of analyses of the highest concentration of anti-factor IIa activity of the RS on the analytical level)





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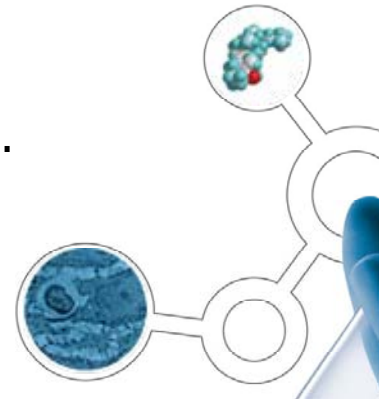
Using the same method for calibration as for the normal day to day analyses will secure that any changes that have an impact on your enzymatic chromogenic assay occurred during maintenance/repair will be discovered. This is not the case if you use a potassium dichromate solution e.g. provided by the manufacturer of the enzymatic analyzer.

If you are above the limit for the RSD% you should investigate until the root cause is found and then set up corrective and preventive actions.

Probable cause for RSD% above limit on an enzymatic analyzer:

- Pipetting problems (e.g. torn syringes/needles)
- Spectrophotometric problems (e.g. fluctuations in absorbance readings)
- Mixing problems (e.g. the mixing paddle is creating air bubbles)
- Incubator problems (e.g. air circulation is not functioning)

Each area has to be thoroughly investigated until the cause is found.



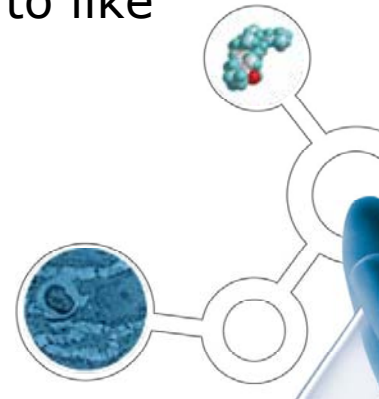


## Enzymatic Chromogenic Assays – LMW-Heparin Achieving and Maintaining Reproducibility and Continuity

If you are outside the limit of the absorbance level the probable cause could be:

- Pipetting problems (e.g. the reagent syringe is torn)
- Spectrophotometric problems
- New lot of reagents or change in reagents

A change in the absorbance level is not as critical as an RSD% outside the limit. This is because the RS is included in each run. E.g. if a change in a reagent occurs it will have the same impact on the absorbance readings of the RS as for the samples. However, this is only true if the RS and the sample are “like to like” molecules.



# Enzymatic Chromogenic Assays – LMW-Heparin Achieving and Maintaining Reproducibility and Continuity

## Range and Linearity

Fig. 4 and 5 show examples of an anti-factor IIa assay (4+2 parallel line assay). As seen it is possible to develop an assay with good linearity throughout the range of  $\pm 20\%$  relative to the RS. (Response =  $\Delta\text{mAbs./min.}$ )

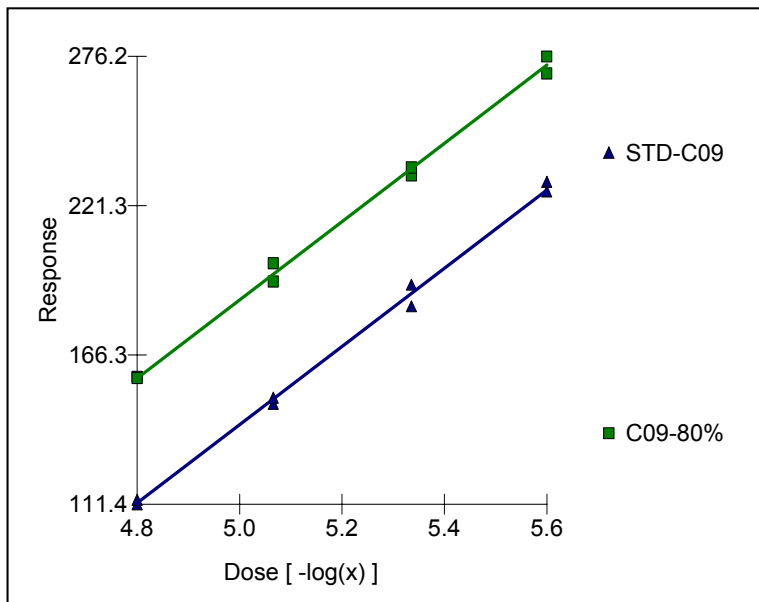


Fig. 4:

STD-C09 = tinzaparin sodium RS (blue line)

C09-80% = 80% dilution of RS (green line)

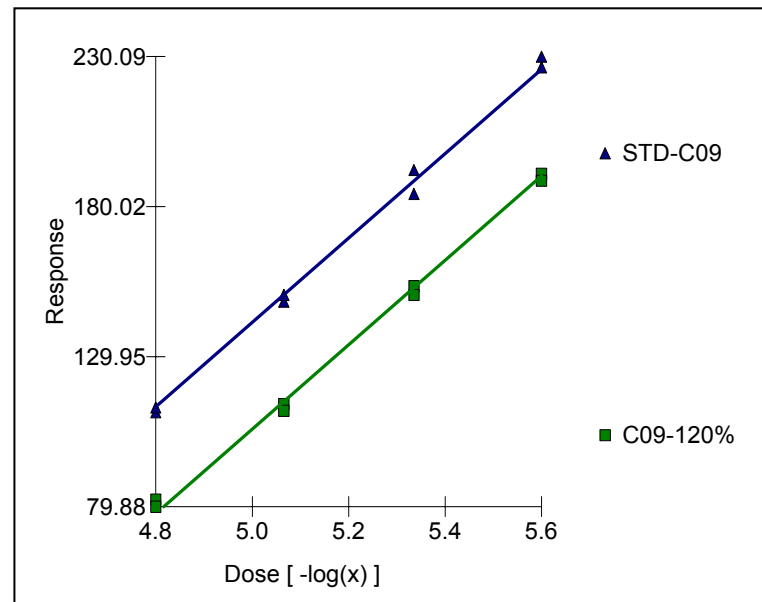
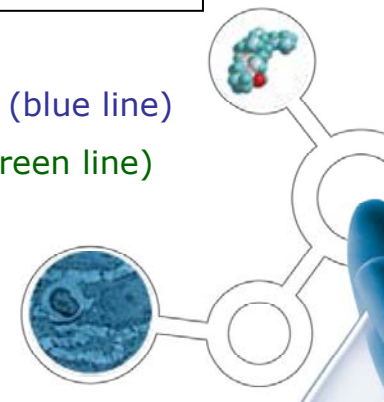


Fig. 5:

STD-C09 = tinzaparin sodium RS (blue line)

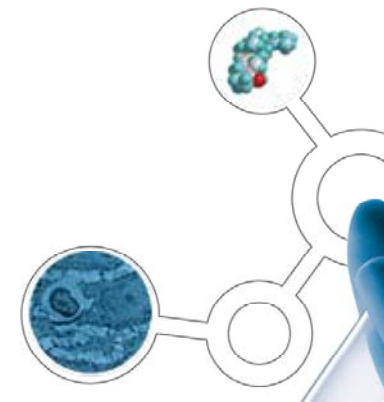
Sample = 120% dilution of RS (green line)



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Table 2: Results from range and linearity study

Sample	80% of RS	120% of RS	RS
Coefficient of Correlation (R <sup>2</sup> ) y=ax+b	0.997	0.998	0.997
Relative Potency	0.801	1.194	n/a
Recovery	100.1%	99.4%	n/a
95%-CI	98.4% - 101.6%	98.4% - 101.7%	n/a



# Enzymatic Chromogenic Assays – LMW-Heparin Achieving and Maintaining Reproducibility and Continuity

## System Suitability Test

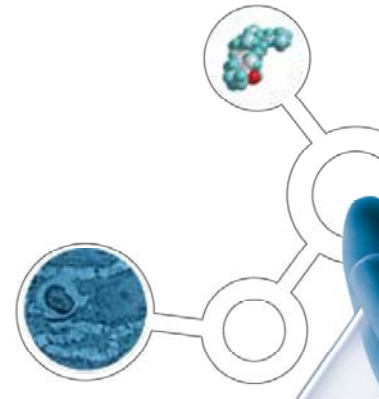
In the following the anti-factor IIa assay is used as an example, however the thoughts and ideas applies for anti-factor Xa assay as well.

The reactions that take place in the anti-factor IIa assay are as follows:

LMW-heparin + ATIII (excess) → LMW-heparin-ATIII

LMW-heparin-ATIII + FIIa (excess) → LMW-heparin-ATIII-FIIa + FIIa (residual)

FIIa (residual) + Chromogenic substrate → peptide + pNA





## Enzymatic Chromogenic Assays – LMW-Heparin Achieving and Maintaining Reproducibility and Continuity

Controlling function of reagents:

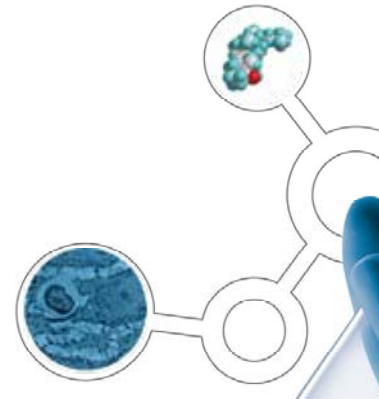
Set up limits for the absorbance readings of

- The highest concentration of your RS on the analytical level.
- The blank solution

If you only set up limits for the blank solution you will not discover if the binding of LMW-heparin to AT-III or the binding of LMW-heparin\*AT-III complex to thrombin (FIIa) has occurred as it should.

Controlling variation:

Set up limits for the 95%-Confidence Intervals (95%-CI) of each determination. E.g.: Maximum 95%-CI = 97% - 103%





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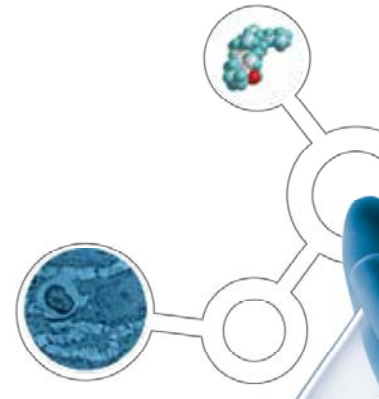
### Stability of reagents

It is important to address both the in-use stability and the long term stability of reagents. Especially the reagents of human or bovine origin are fragile.

Test the in-use stability of reagents by setting up a repeatability study.

Test the long term stability of reagent solutions by setting up a repeatability study on e.g. day 1, day 7, day 14 from the manufacturing date of the solutions.

If no upwards or downwards trend in the absorbance readings are seen the reagents are stable.



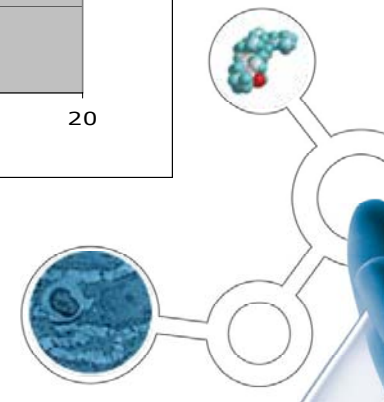
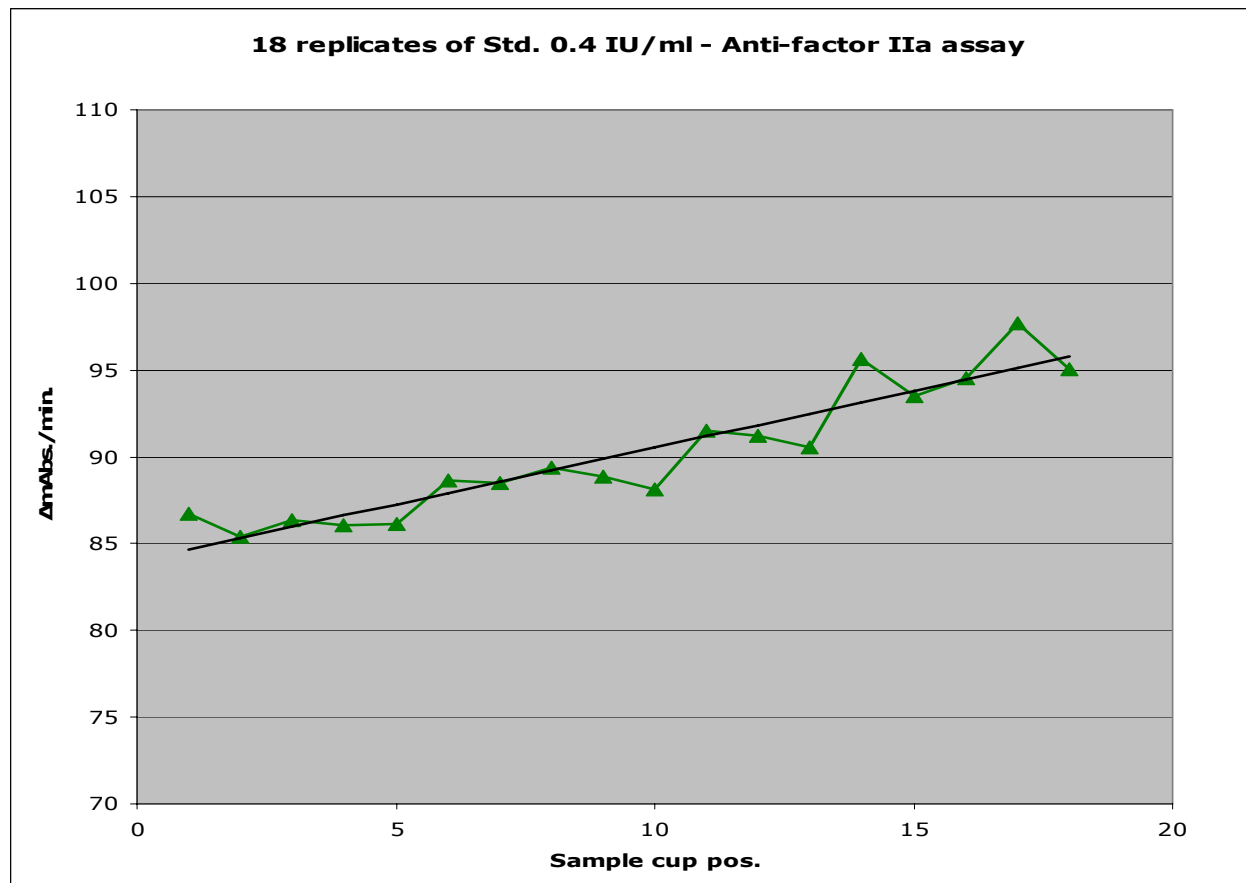
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An example of in-use instability of reagents:

Fig. 6:

18 replicates of tinzaparin sodium RS std. 0.4 IU/ml were analysed.

A considerable upward trend is seen in the absorbance readings from sample cup 1 to 18 indicating in-use instability of reagents.

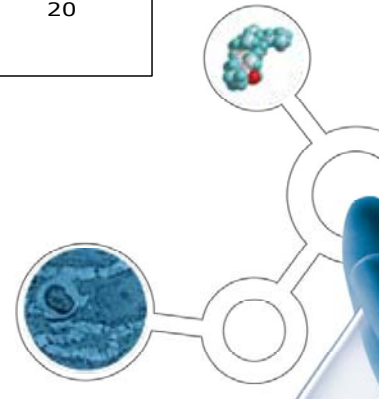
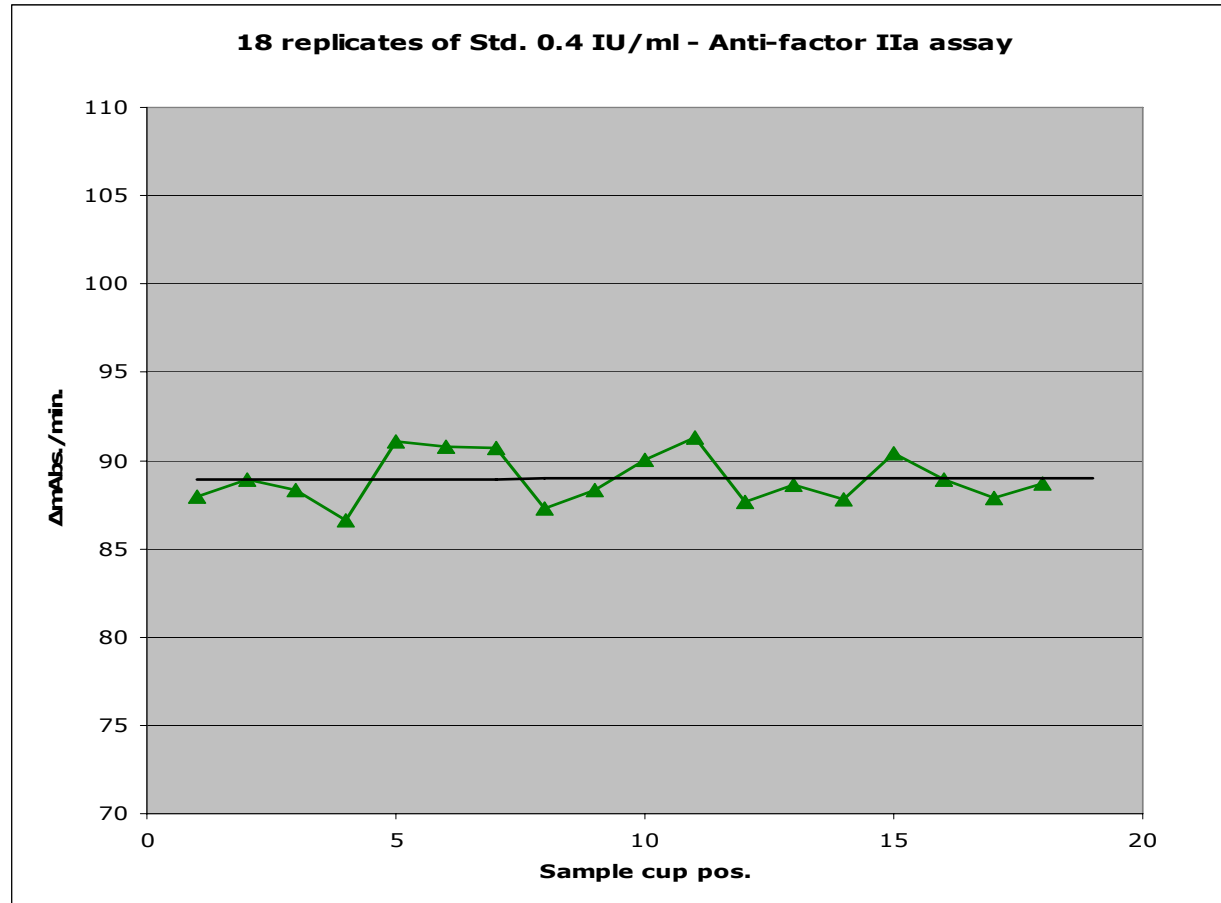


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Fig. 7:

The solution to the instability problem was to use a different kind of reagent cups equipped with a lid.

The absorbance readings from sample cup 1 to 18 are now stable.



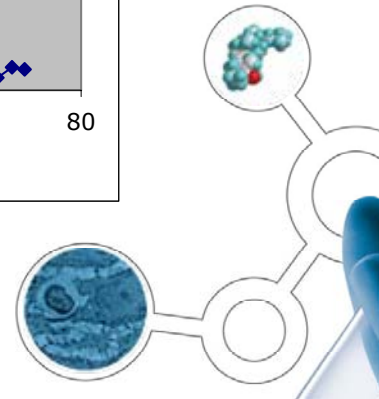
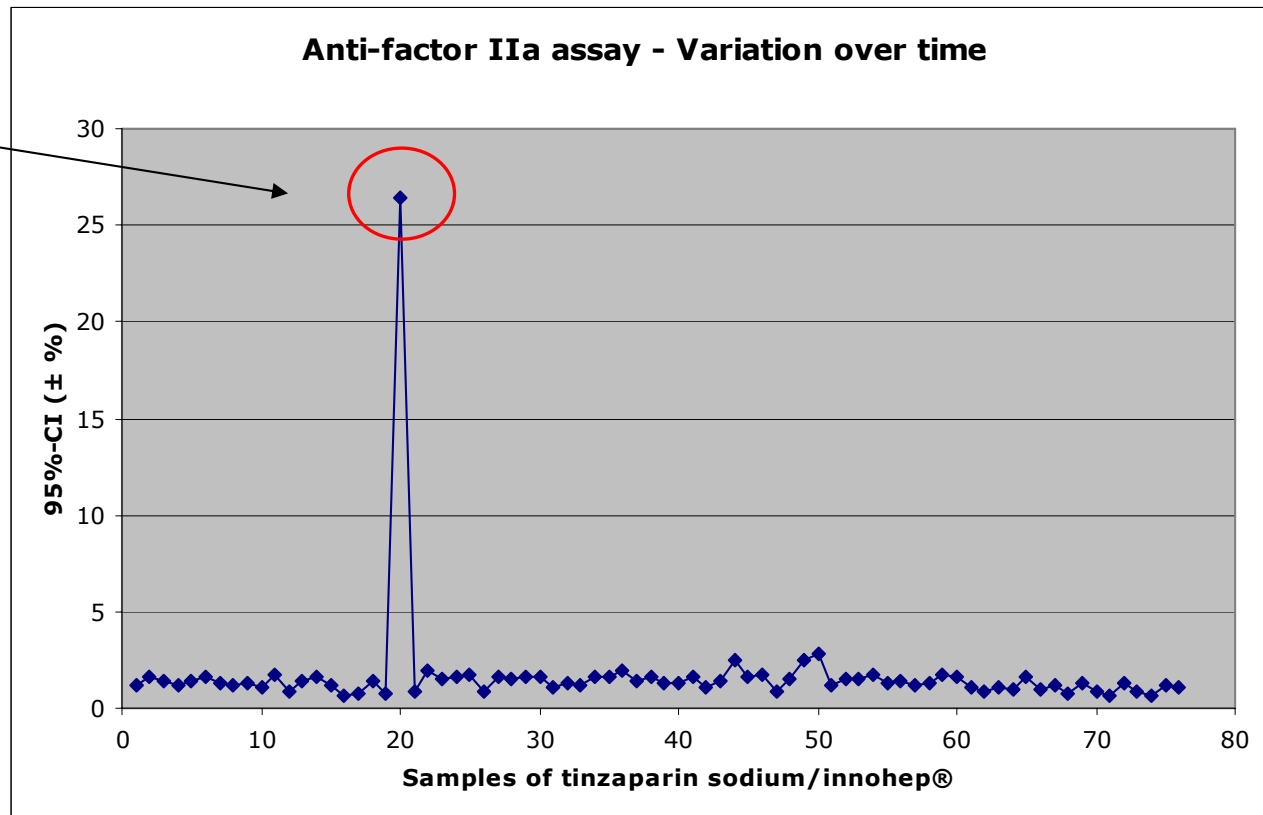
# Enzymatic Chromogenic Assays – LMW-Heparin Achieving and Maintaining Reproducibility and Continuity

## Monitoring of assay variation

In order to control assay variation over time it is a good idea to create assay variation control charts such as the one shown in Fig. 8. Remember to log OOT responses incl. the identified cause.

Cause:  
Human  
error,  
dilution  
mix-up.

Fig. 8:  
95%-CI  
(±%) from  
the anti-  
factor IIa  
assay of  
samples of  
tinzaparin  
sodium and  
**innohep®**





## Enzymatic Chromogenic Assays – LMW-Heparin Achieving and Maintaining Reproducibility and Continuity

### Investigation of OOT responses

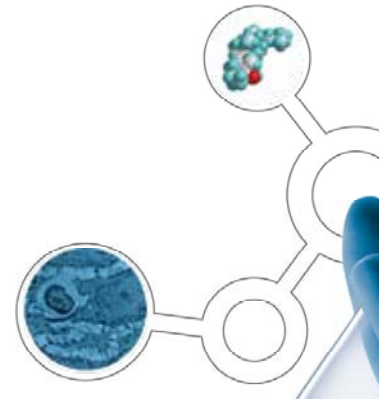
OOT responses should be investigated thoroughly until the root cause is found.

Example of an OOT investigation:

The RSD% of the Anti-factor Xa calibration test of the enzymatic analyzer did not comply with limit:

RSD% = 5.5%

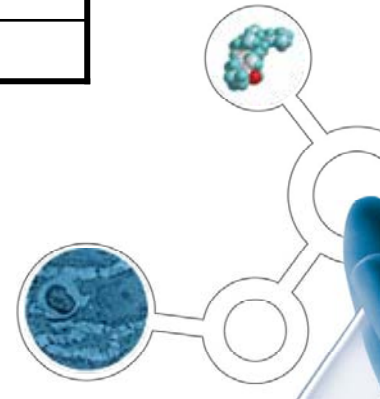
Limit: RSD%  $\leq$  2.5%



## Enzymatic Chromogenic Assays – LMW-Heparin Achieving and Maintaining Reproducibility and Continuity

Table 3: Results from Anti-factor Xa calibration test. The limit for RSD% is not met (Limit:  $RSD\% \leq 2.5\%$ )

Determination No.	$\Delta mAbs./min.$
1	96.50
2	93.25
3	94.05
4	97.26
5	95.89
6	82.39
7	86.85
8	97.72
9	95.04
10	88.35
SD	5.14
RSD%	5.5
Mean	92.73

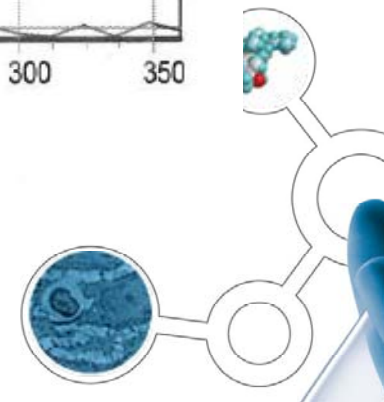
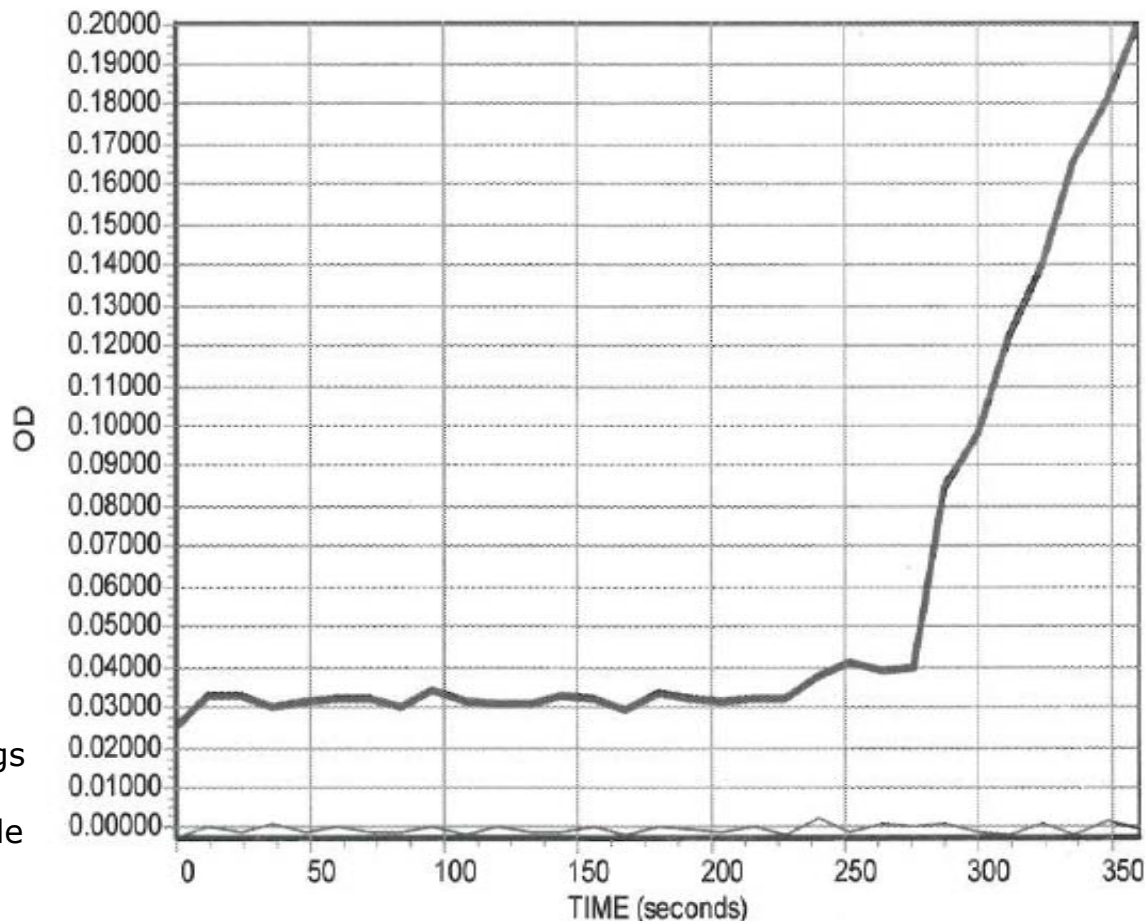


## Enzymatic Chromogenic Assays – LMW-Heparin Achieving and Maintaining Reproducibility and Continuity

After ruling out pipetting, mixing and/or reagent problems we looked at the spectrophotometer raw data.

The absorbance readings over time fluctuated and the background absorbance was higher than usual (around 0.03). This could explain the high RSD% in our Anti-factor Xa calibration test.

Fig. 9: Absorbance readings (405 nm) over time from determination no. 8 in table 3, sample: tinzaparin sodium RS 0.42 IU/ml of Anti-factor Xa activity

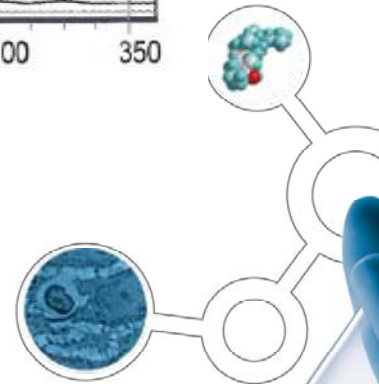
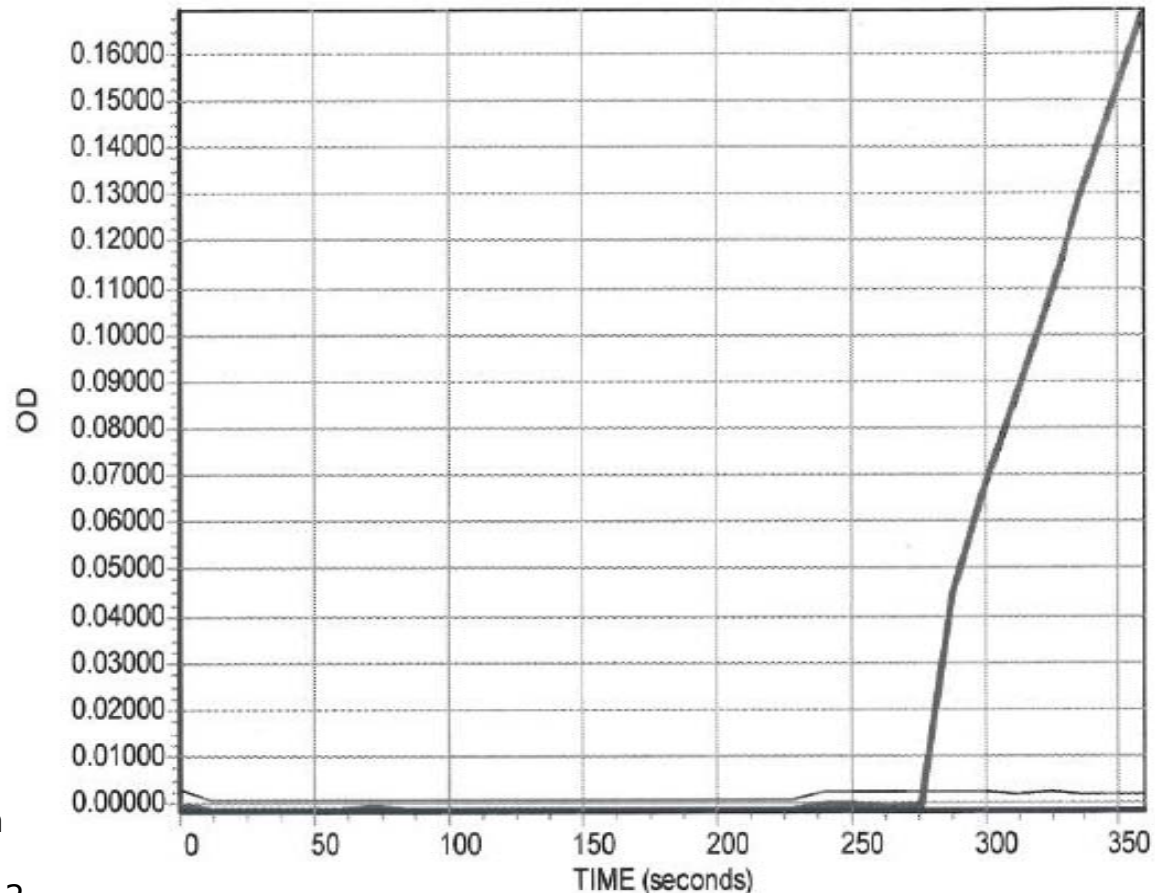


# Enzymatic Chromogenic Assays – LMW-Heparin Achieving and Maintaining Reproducibility and Continuity

As a consequence of the poor spectrophotometer readings we replaced both the spectrophotometer and the cuvette rotor motor.

Afterwards the absorbance readings were stable and the background absorbance were back to normal (around 0.0)

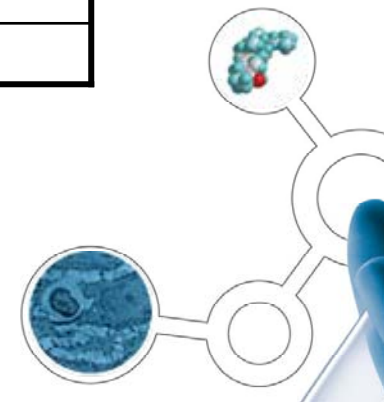
Fig. 10: Absorbance readings (405 nm) over time from determination no. 9 in table 4, sample: tinzaparin sodium RS 0.42 IU/ml of Anti-factor Xa activity



## Enzymatic Chromogenic Assays – LMW-Heparin Achieving and Maintaining Reproducibility and Continuity

Table 4: Results from Anti-factor Xa calibration test. The limit for RSD is now met (Limit:  $RSD\% \leq 2.5\%$ )

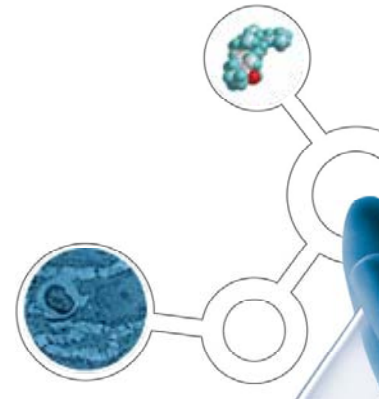
Determination No.	$\Delta mAbs./min.$
1	101.41
2	100.03
3	102.63
4	99.36
5	100.95
6	102.21
7	103.15
8	98.52
9	103.62
10	101.49
SD	1.60
RSD%	1.6
Mean	101.30





## Enzymatic Chromogenic Assays – LMW-Heparin Achieving and Maintaining Reproducibility and Continuity

After this incident we decided to print out the absorbance readings over time when calibrating the enzymatic analyzer. In this way we are able to discover changes in the stability of the absorbance readings and take proper action before the RSD% limit of the calibration test is not met.



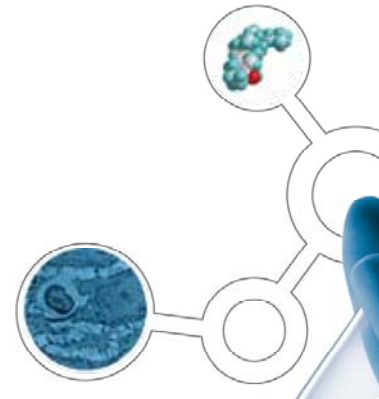


## Enzymatic Chromogenic Assays – LMW-Heparin Achieving and Maintaining Reproducibility and Continuity

### Testing new lots of reagents

An important factor in securing continuity in enzymatic chromogenic assays is the acceptance testing of new lots of reagents.

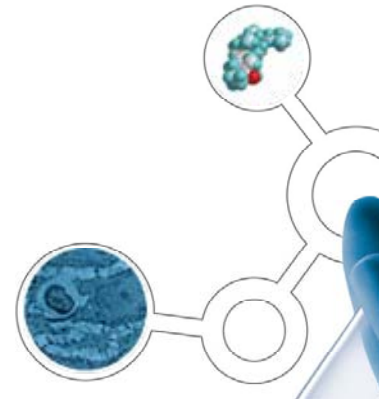
An example of this is the acceptance testing of new lots of Factor Xa reagent:



## Enzymatic Chromogenic Assays – LMW-Heparin Achieving and Maintaining Reproducibility and Continuity

Results from the testing of new lots of Factor Xa reagent:

Lot Factor Xa	Working solution of FXa (nkat/ml)	Absorbance Blank solution ( $\Delta$ mAbs./min.)	Absorbance tinzaparin sodium RS 0.4 IU/ml ( $\Delta$ mAbs./min.)
A	10	277	77
B	10	312	105
C	10	306	109
D	10	270	86

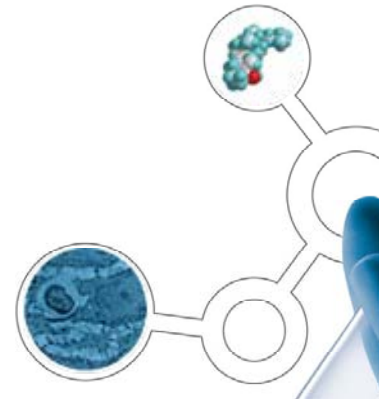




## Enzymatic Chromogenic Assays – LMW-Heparin Achieving and Maintaining Reproducibility and Continuity

The results show that both the absorbance readings of the blank solution and of the tinzaparin sodium RS 0.4 IU/ml solution differ even though the activity of the working solutions of Factor Xa are the same (10 nkat/ml).

The results show that in order to secure continuity it is important to test new lots of Factor Xa.



# Enzymatic Chromogenic Assays – LMW-Heparin Achieving and Maintaining Reproducibility and Continuity

## Results from QC-analyses of tinzaparin sodium

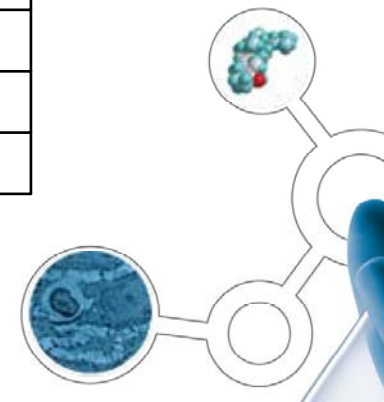
Anti-factor IIa results from the four most recently manufactured lots of tinzaparin sodium (four determinations and mean). It is observed that all 95%-Confidence Intervals are within 97% - 103%.

Lot X	Anti-factor IIa Activity (IU/mg)	95%-Confidence Interval (%)
1	59.31	99.0 – 101.0
2	60.41	98.4 – 101.6
3	59.98	99.2 – 100.8
4	61.14	98.9 – 101.1
Mean	60.2	99.2 – 100.8

Lot C	Anti-factor IIa Activity (IU/mg)	95%-Confidence Interval (%)
1	58.35	98.9 – 101.1
2	58.43	98.9 – 101.1
3	59.29	99.1 – 100.9
4	59.20	98.7 – 101.3
Mean	58.8	99.3 – 100.7

Lot	Anti-factor IIa Activity (IU/mg)	95%-Confidence Interval (%)
1	55.17	98.6 – 101.4
2	55.35	98.5 – 101.5
3	56.14	98.7 – 101.3
4	56.08	98.9 – 101.1
Mean	55.8	99.4 – 100.6

Lot D	Anti-factor IIa Activity (IU/mg)	95%-Confidence Interval (%)
1	62.19	98.8 – 101.2
2	62.35	98.5 – 101.5
3	62.13	97.2 – 102.8
4	61.66	98.8 – 101.2
Mean	62.0	99.4 – 100.4



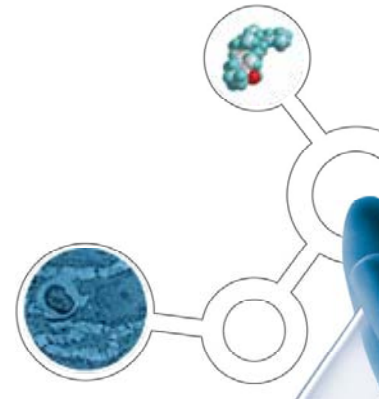


# Enzymatic Chromogenic Assays – LMW-Heparin Achieving and Maintaining Reproducibility and Continuity

## Conclusions

In order to achieve and maintain reproducibility and continuity in enzymatic chromogenic assays it is essential to:

- Use an automated enzymatic analyzer preferably equipped with a build-in incubator and air circulation.
- Use the principles of your normal day to day assay for the calibration test of your enzymatic analyzer. Set up limits for the RSD% and the absorbance level.
- Linearity must be present throughout the total range
- Set up a system suitability test with limits for the absorbance level of the blank as well as of the highest concentration of your RS on the analytical level. Also set up limits for the 95%-Confidence Intervals of your single assay determinations.
- Test the in-use stability as well as the long term stability of reagents by setting up repeatability studies. No downwards or upwards trend must be present.
- Monitor assay variation over time. Log OOT responses and the identified cause.
- Investigate OOT responses until the root cause has been found.
- Perform acceptance testing of new lots of reagents



# Enzymatic Chromogenic Assays – LMW-Heparin Achieving and Maintaining Reproducibility and Continuity



The enzymatic chromogenic team at LEO Pharma  
B. Fehrmann and staff

