Case studies of non-parallelism in various ELISAs

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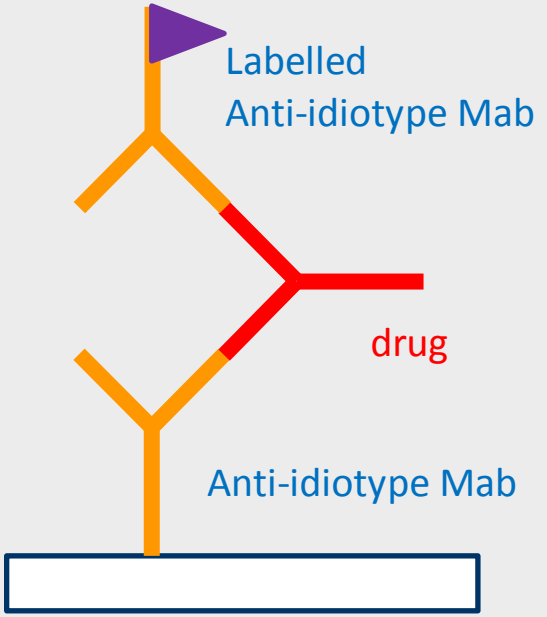
Case study 1 - Non-parallelism of a Pk ELISA (from 2000)



1. Bridging Pk ELISA for a therapeutic antibody, developed and successfully validated (individual spike recovery = **matrix effect ok in healthy volunteers**)
2. First phase I clinical study measured, evaluated and reported
3. Parallelism experiment performed with remaining study patient samples after release of study, **parallelism failed!!!**

Case study 1 - Non-parallelism of a Pk ELISA

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4. **Investigations** indicated labile assay conditions regarding individual samples (matrix effects in some patients only)

   a. equilibrium in sample incubation step not reached

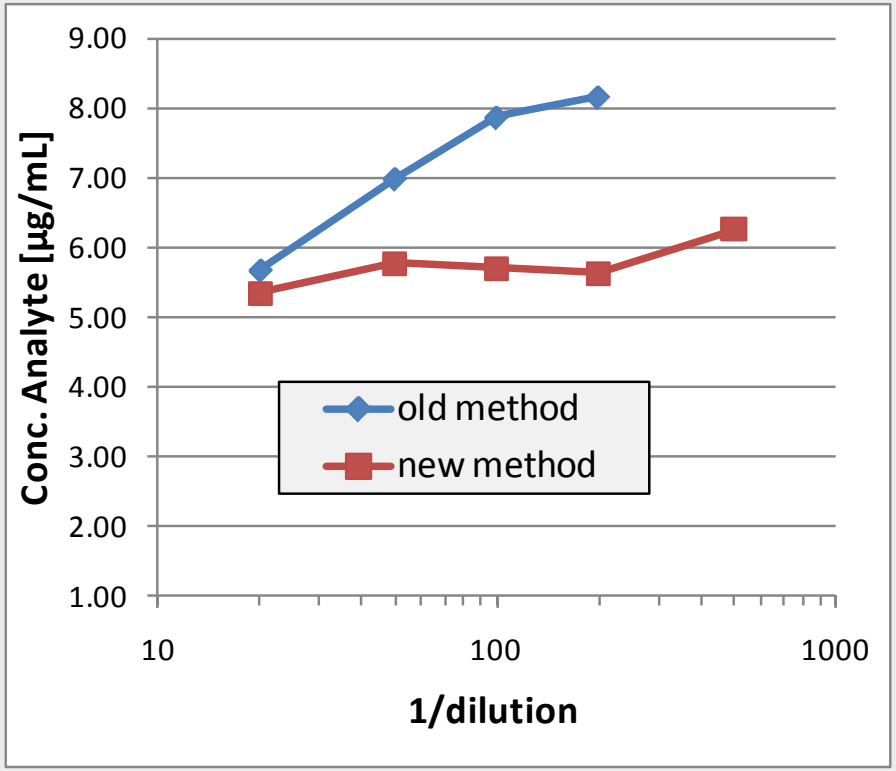
   b. Calibration curve shape differs between preparation in pure or diluted matrix

   c. Assay performance depends significantly on immobilization conditions (sterical hindrance at too high coating levels of capture antibody)

Case study 1 - Non-parallelism of a Pk ELISA

5. **Follow up activities: new method development**
   - prolonged sample incubation time (8h instead of 2h)
   - preparation of twenty fold concentrated calibration standards in pure plasma (100%, MRD 1:20) instead of ready-to-use diluted plasma (5%)
   - batchwise coating of plates including suitability check (shape of curve and signal of Cmax)

6. **Revalidation** of the assay



Case study 1 - Non-parallelism of a Pk ELISA

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5. Repeat analysis of whole study

study sample	first result old method v2.0 [µg/mL]	repeat analysis new method v3.0 [µg/mL]	rel. Dev (%) (diff/mean)
U001#2	24.4	23.3	-4.6%
U002#2	30.4	30.3	-0.3%
U003#2	31.4	31.1	-1.0%
U004#2	27.7	28.3	2.1%
U005#7	39.1	44.1	12.0%
U028#7	45	57.4	24.2%
U030#9	48.2	61.9	24.9%
U030#14	46.7	71.1	***41.4%***
U030#16	48.9	69.7	***35.1%***
U033#5	33.5	31.7	-5.5%
U024#9	17.5	17.3	-1.1%
U002#13	27.1	17.6	***-42.5%***
U029#13	21	9.99	***-71.1%***
		N	13
		mean rel. Dev.	1.0%
		% |rel dev.| > 30%	**30.8%** 4 out of 13

High interindividual variability.
Not good enough!

Case study 1 - Non-parallelism of a Pk ELISA

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5. Second **new result used for repeat evaluation of pharmacokinetics**

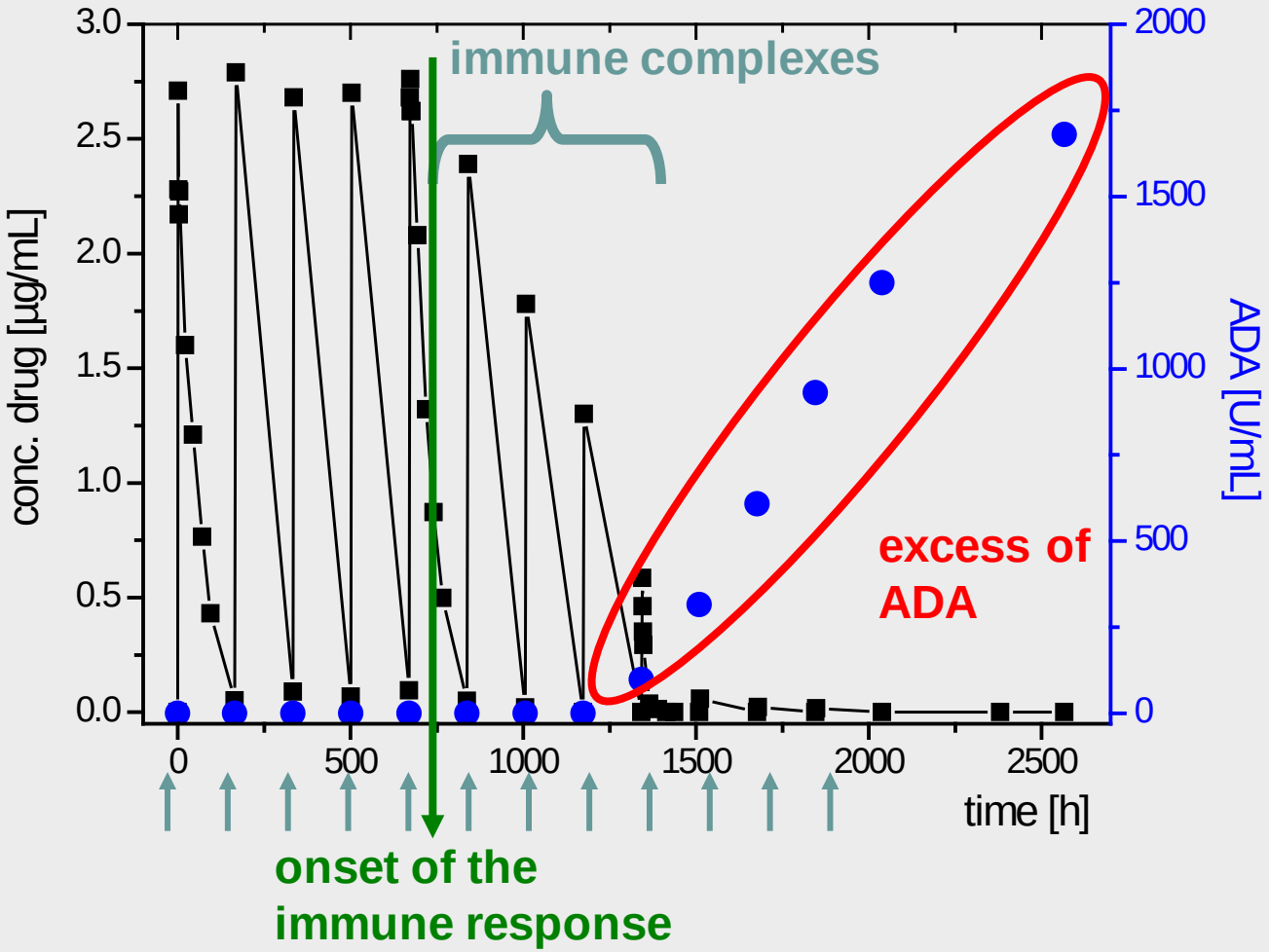
6. **Amendment to the Clinical Trial Report** with revision of the bioanalytical and pharmacokinetic sections

7. **<u>Lessons learned</u>**:

   - Perform the first parallelism test as soon as possible during the ongoing first phase I study!
   - Test for parallelism cannot be replaced by matrix effect (selectivity) experiment.

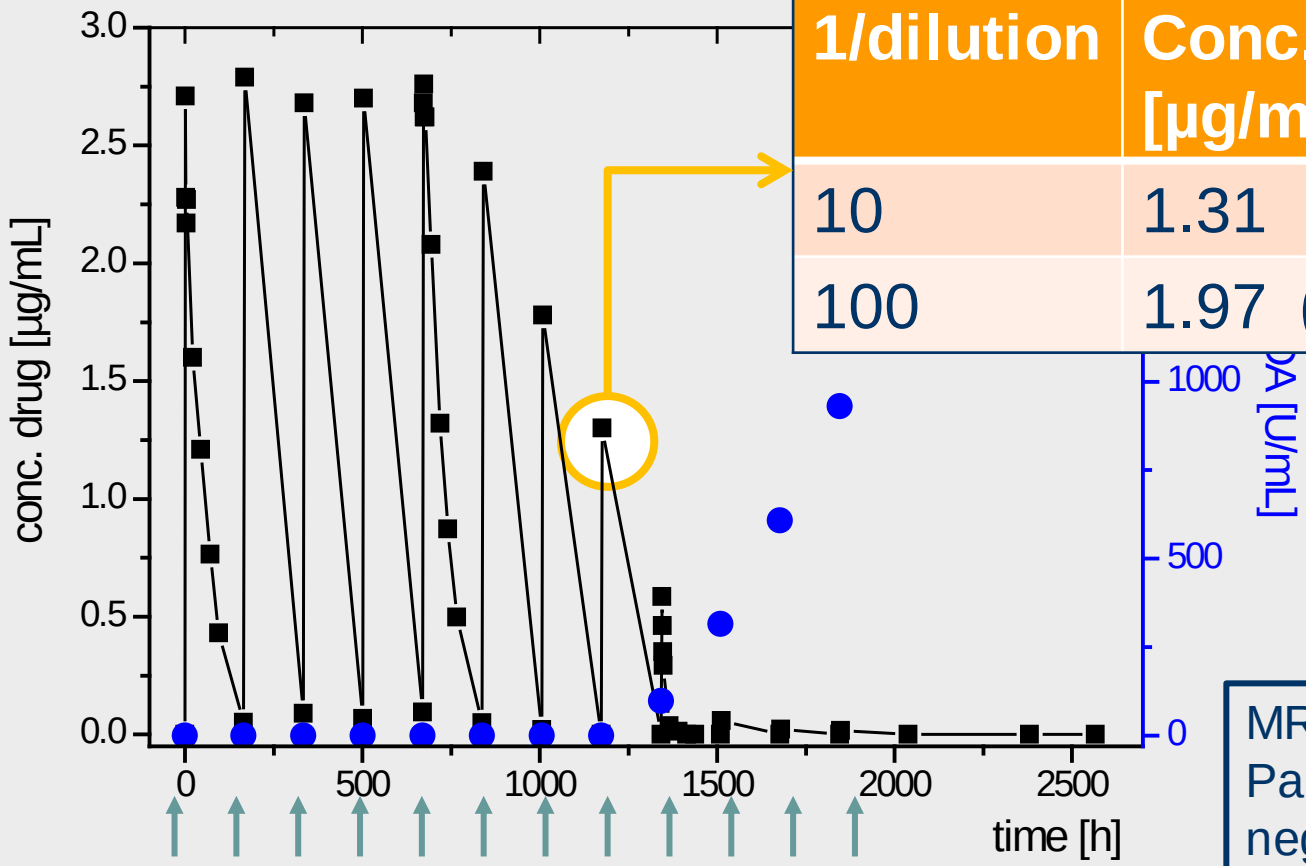
Case Study 2 - Non-parallelism of ADA positive Pk samples

- Pk profile and immune response of a therapeutic antibody in a multi-dose study
- Interference of ADA in the drug ELISA by masking epitopes
- Interference of drug in the ADA bridging ELISA



Case Study 2 - Non-parallelism of ADA positive Pk samples

- **ADA positive pk samples often show non-parallelism** if analyzed in different dilutions due to the polyclonal mixture of ADAs with various affinities (change of equilibria)



1/dilution	Conc. drug [µg/mL]
10	1.31
100	1.97 **(+ 50%)**

MRD = 1:10
Parallelism in ADA negative samples ok

Case Study 2 - Non-parallelism of ADA positive Pk samples

- <u>Lession learnt:</u>
- Samples with proven non-parallelism (measured at various dilutions) should be reported as „**no valid result**“!
- Should we analyze all ADA positive Pk samples in different dilutions to check parallelism? Or one per patient?

- If ADA interfere in the pk drug ELISA
  Then drug concentrations measured in ADA positive samples should be excluded from pharmacokinetic evaluation (no longer total drug conc.)

Case study 3 – endogenous serum biomarker (commercial research grade ELISA kit)

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Relative deviation from 1/100 reference dilution

Dilution 1:	patient # 130289 S	126738 S	3377 P	98781 S	101850 S	A01 S	A 02 S	6044 P	102730 P	101111 P
5			79%							
10		47%								
20		49%	53%		70%	22%			44%	27%
50	53%	15%	19%	11%	34%	55%	23%	14%	27%	7%
100	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
200	-10%			-24%	-14%		0%	-28%		-13%
500	-25%									

Reference dilution

Linear regression after log-log transformation, slope indicates trend conc. vs. dilution

Dilution 1:	patient # 130289 S	126738 S	3377 P	98781 S	101850 S	A01 S	A 02 S	6044 P	102730 P	101111 P	mean slope	CV slope
5			531									
10		1460										
20		1480	455		882	496		1100	720	1150		
50	6780	1150	352	2580	694	631	3980	918	638	967		
100	4430	996	297	2330	519	407	3240	803	501	902		
200	3980			1780	446		3230	578		789		
500	3340											
slope	1.289	1.181	1.196	1.268	1.307	1.103	1.151	1.268	1.220	1.158	1.214	5.6%
r^2	0.994	0.998	0.999	0.997	0.999	0.950	0.995	0.997	0.998	1.000		

Slopes fail acceptance criterion: not parallel

U. Kunz, 2013

Case study 3 – endogenous serum biomarker

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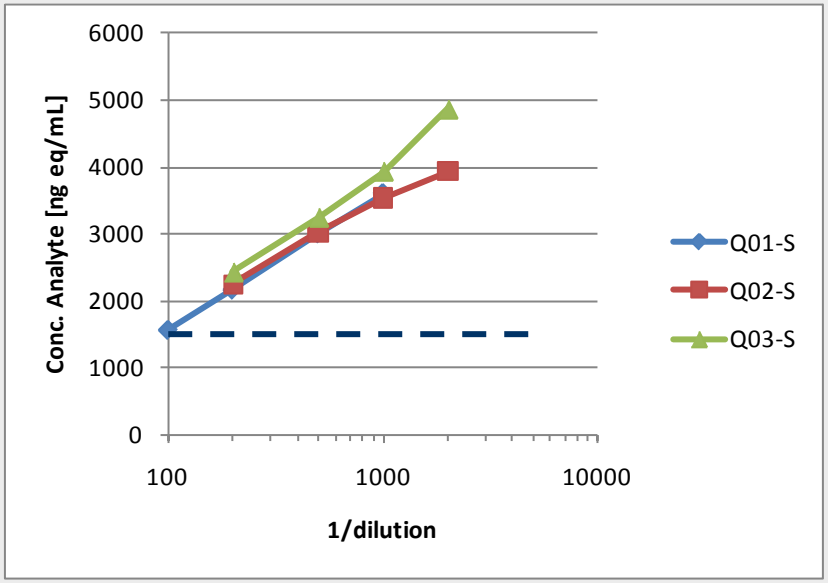
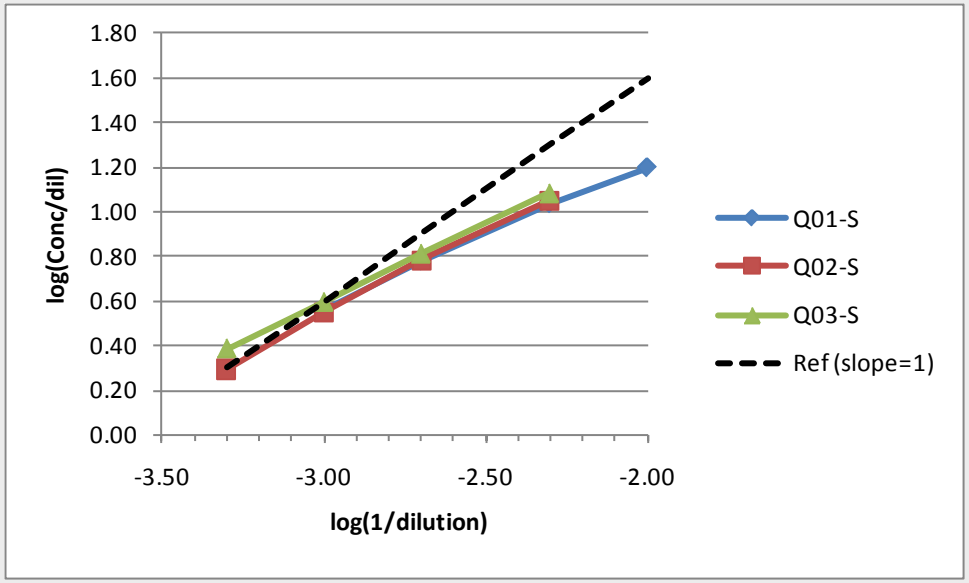
1. Parallelism experiment failed!
2. Consequence: no longer a relative quantitative assay, no valid concentration values
3. Discussion of possible options with client:
   - Alternative ELISA with other antibodies, other reference standard?
     -> **Not available** within timeline.
   - Relative quantitative with fixed dilution?
     -> **No option,** huge range of endogenous levels. Furthermore, scientifically questionable.
   - **Quasi-quantitative assay** (post/pre-dose)?
     problem: huge range of endogenous levels requires individual dilutions of patient samples!
4. Solution: Validation of a quasi-quantitative assay with **titer determination of pre-dose samples + post/pre-dose ratios for the post-dose samples at the individual, optimal dilution**

Case study 4 - Non-parallelism of an **in-vitro diagnostic** assay for a clinical relevant endogenous biomarker





	Q01-S	Q02-S	Q03-S	
CV (N=4 dilutions)	35 %	23 %	29 %	≤30%?
Slope (after log-log transformation)	0.64	0.76	0.7	0.85≤slope ≤1.15?
R^2 (coeff. of determin.)	0.996	0.997	1.0	>0.98?

Case study 4 - Non-parallelism of an **in-vitro diagnostic** assay

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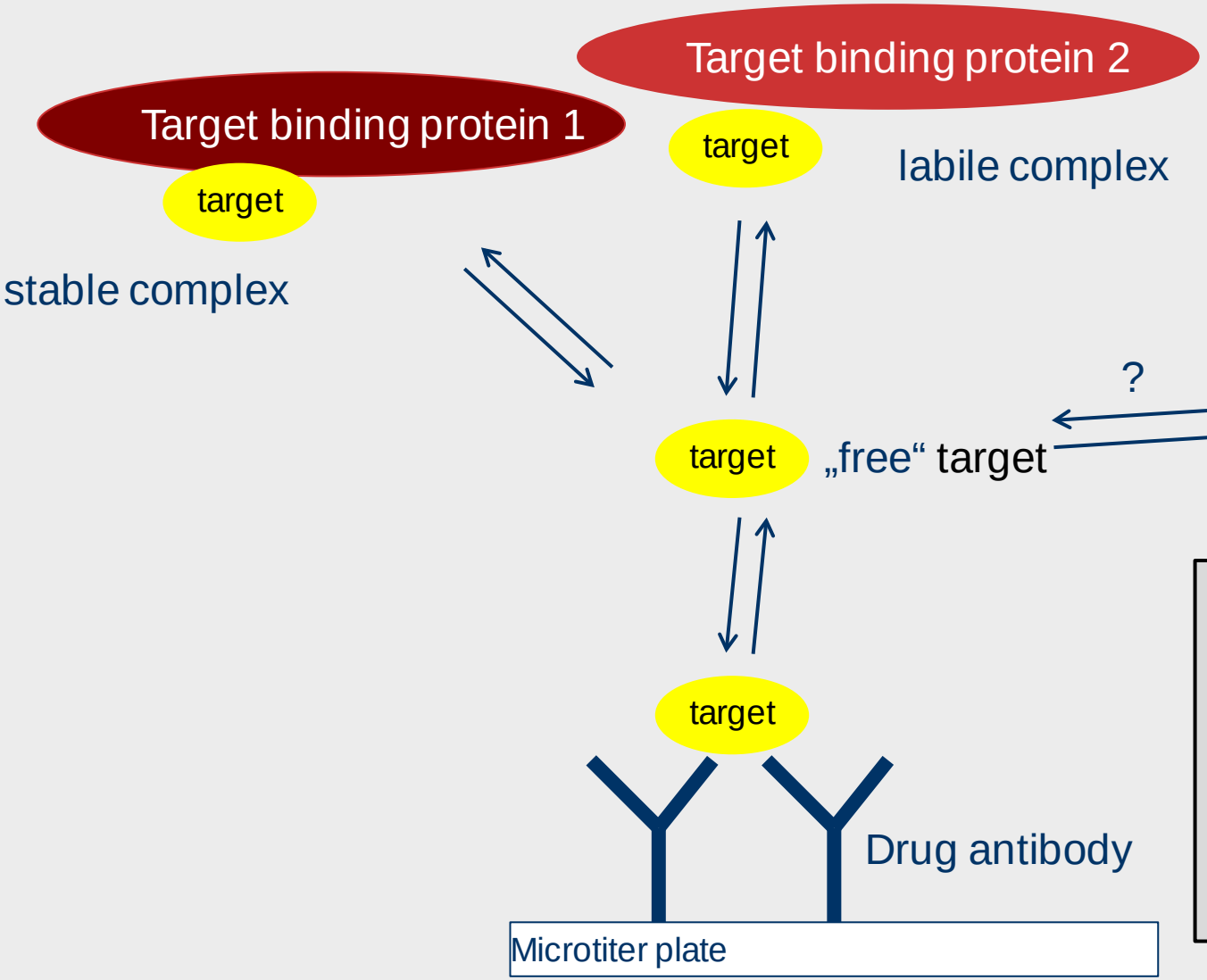
1. Parallelism experiment failed!
   In contrast to the kit manual!
2. The ELISA kit of this particular lot is not suited for relative quantification of the clinical relevant biomarker and therefore not suited for its intended use!!!
3. Manufacturer was contacted and confrontated with our results
4. Manufacturer confirmed trouble with the kit (due to change in reagents); unknown if kit was taken from the market
5. Manufacturer is developing a new version of the kit
6. BI decided not to wait for the new version but to switch to an alternative provider

Lesson learnt: Do not trust in kit manuals, test by yourself.
Kit performance may change between lots.

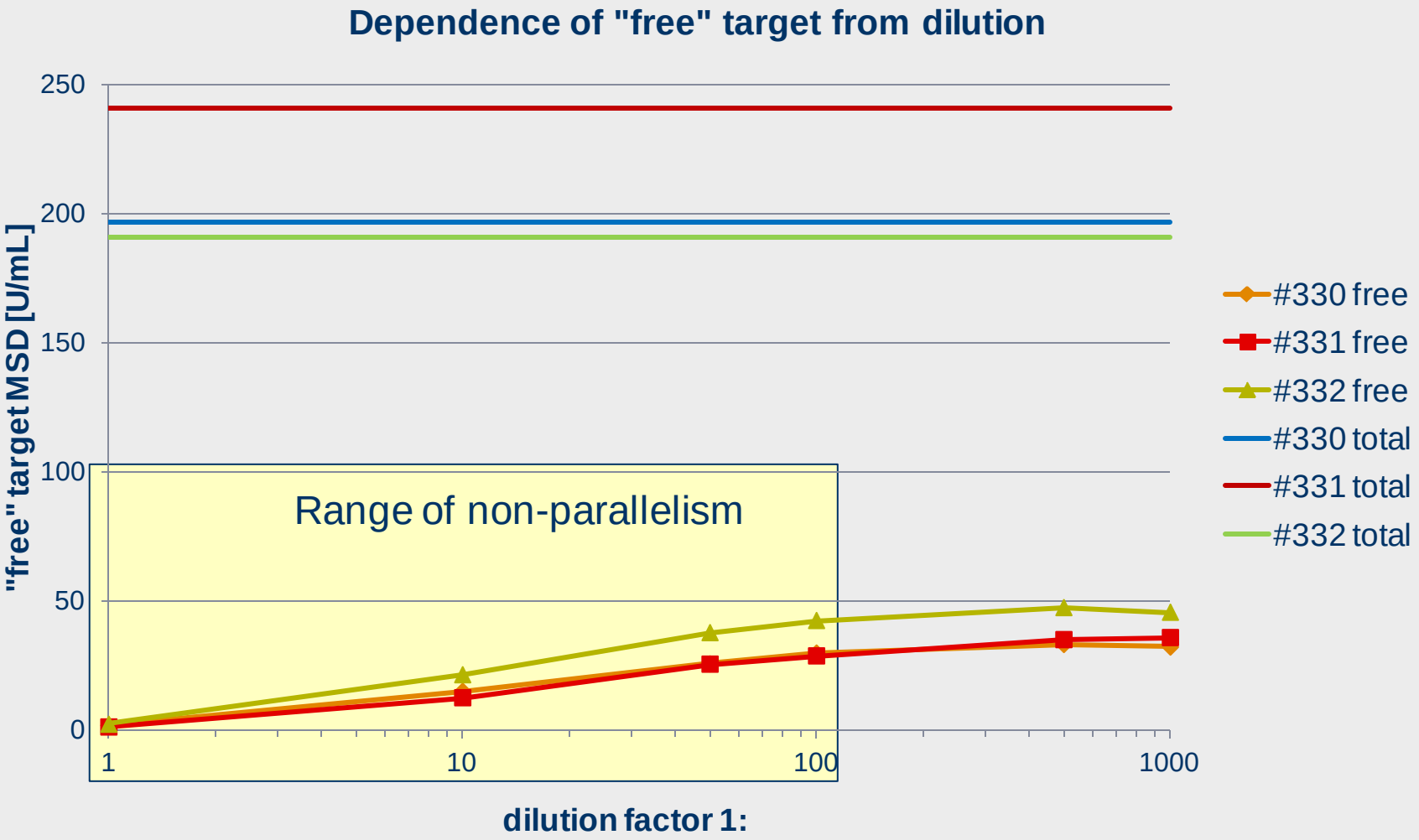
Case study 5 - „free“ target immunoassay



Parameters that affect equilibria:

- Temperature
- pH
- Ionic strength
- Buffer compounds
- **Sample dilution**

Case study 5 - Dilution of samples affect measurable target fraction



Case study 5 - „free“ target immunoassay

- A more sensitive MSD assay was chosen instead of the previous ELISA in order to enable a **minimum required dilution of 1:300, outside the range of non-parallelism**
- Assay quantifies the dissociable/targetable fraction of target not the „free“ fraction

General questions raised:

- How to check parallelism in „free“ target/drug assays when sample dilution alters the equilibria and no plateau can be reached?
- How to validate such an assay?
- Are these assays **quasi-quantitative by definition**?
  Should we report ratios instead of concentrations?

Summary

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Non-parallelism occurs:

- rarely in pk drug ELISAs (specially therapeutic antibodies), one example only within EBF community so far!
- most often in ADA positive samples if ADAs interfere in the pk ELISA
- frequently in research grade ELISA kits for endogenous protein biomarker
- sometimes in new lots of kits after change of reagent lots
- in the determination of „free“ fractions of targets, drugs or biomarker; dependent on affinities of interaction equilibria

Summary - Reasons for non-parallelism

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- **Unspecific matrix effects** may affect binding of analyte to assay antibodies Could be individually different (see case study 1).
- **Differences** in the immunoreactivity **between endogenous analyte and reference standard** (most probable case study 3, maybe 4)
  e.g. due to structural differences (folding, multimerisation), post-translatorical modifications (glycosilation pattern, sulphatation, phosphorylation)
- **Change of equilibria** in matrix sample by dilution, e.g.
  - Release of analyte from labile complexes (see case studies 2 and 5)
  - refolding of analyte due to changes of the surrounding (less protein, buffer instead of matrix)
  - aggregation/de-aggregation
- Other?

If parallelism experiment is successful…

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A successful parallelism experiment is the prove for:

- **Absence of unspecific matrix effects** within dilution range (possibility of extending the range of measurement by higher pre-dilutions of samples)
- **Comparability of reference standard with endogenous analyte** (Biomarker), in the particular immunoassay
- **Stable equilibrium** between analyte and interacting proteins or measurement of total analyte.

If parallelism experiment failed…

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A failed parallelism experiment is the prove for:

- **Nothing!** (see „Reasons for non-parallelism“)
- Further experiments may be necessary to investigate non-parallelism
- How to proceed:
  - develop a new assay with other reagents, reference standard, buffer, assay conditions….
  - or validate the assay as quasi-quantitative (no concentrations)!

Parallelism testing:

- is one of the most important experiments for quantitative immunoassays!
- should be performed as early as possible during method development or as soon as adequate test samples are available.

Acknowledgement

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