Case Study 12 – A total soluble target ELISA – translating CoU into an analytical strategy and a validation plan

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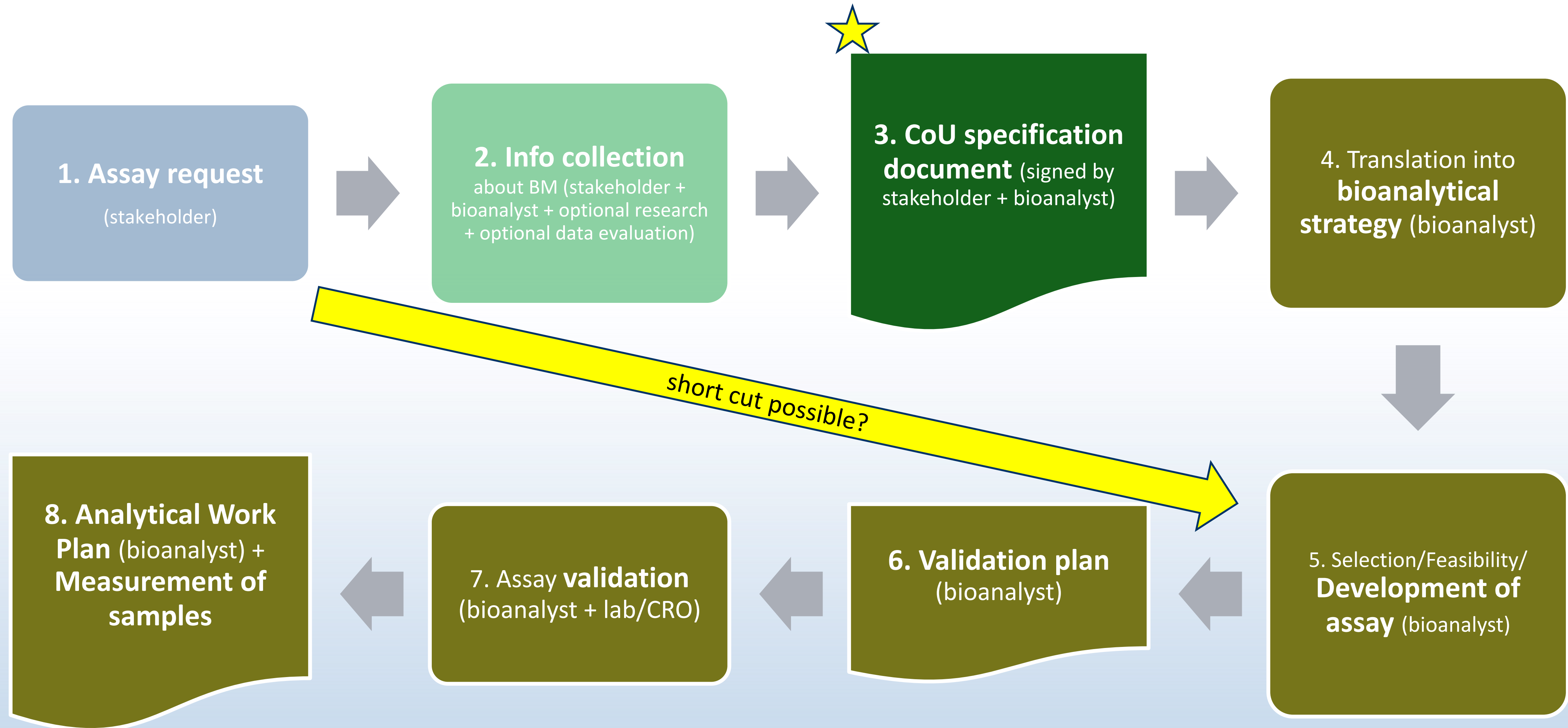
Overview – from assay request to sample measurement



CoU – total Angiopoietin-2_O15123

3. CoU specification document (signed by stakeholder + bioanalyst)

- BM category: indirect target engagement pd biomarker (**explorative** = not clin. qual.)
- BM purpose: **Proof of target engagement (proof of pharmaceutical principle)**
  optional: test for predictive potential of BM
- Stage of drug development: Phase I-II
- Trial population: **patients** (oncology)
- biological context: **soluble target of drug (antagonist),** blocking of target from receptor stops angiogenetic signal cascade
- Expected magnitude of biomarker change to affect decisions:
  strong increase after treatment, unknown extent, half-life prolongation due to target-drug complex formation, possible feed back mechanism unknown
- BM impact on drug development: **supports dose selection**, together with pk and safety
- Risk (patient, regulatory, business): **middle** (all 6 questions = no, 2 = yes, business, regulatory)
- Data evaluation: **pk/pd modelling** (total target, “free” target, total drug)
- Data comparability: long-term comparability multiple studies (**5-6a**)
- Frequent interim data evaluations to accelerate drug development

Translation into a bioanalytical strategy – assay requirements

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Specificity: total Ang-2
No interference of drug and sTie-2 (receptor)

Relative quantitative assay = proven parallelism (>2 logs)

LLOQ < healthy volunteer level

Between-run Precision < longitudinal biological variation

Robust sampling conditions, comprehensive stability investigations

Banking of reference standard, monitoring of assay performance, bridging of QC lots, revalidations

Translation into a bioanalytical strategy – assay development plan

4. Translation into **bioanalytical strategy** (bioanalyst)

- **Feasibility of commercial ELISA kits** (2 kits each), reliable vendors,, **LLOQ < healthy level,** acceptable analytical performance (cal curve), **no or saturable drug interference, parallelism**
- **If not successful** screening of antibodies and **own assay development**
- **Selection of calibration standard** (rec. FC dimer vs monomer)
- **Banking of bulk reference standard and critical reagents**
- **Start stability investigation on standard stock and sub-stock solutions, tracking of lots**
- Optimization of assay conditions, robustness vs. incubator, manual vs. automated processing (robot)
- **Screening of healthy volunteer samples**, verifying matrix (plasma vs. serum), selection of validation samples/QCs, =anchor for identification of long-term trends
- **Longitudinal biological variance** using banked HV samples
- Set of precision criteria
- **Sampling stability** by use of fresh blood
- Set-up of assay monitoring and bridging concept (QC charts, tracking of reagent and QC lots)
- Banking of monitoring matrix samples

Goal: keeping the assay under control over several years
-> generating comparable results for the stakeholder

Validation plan

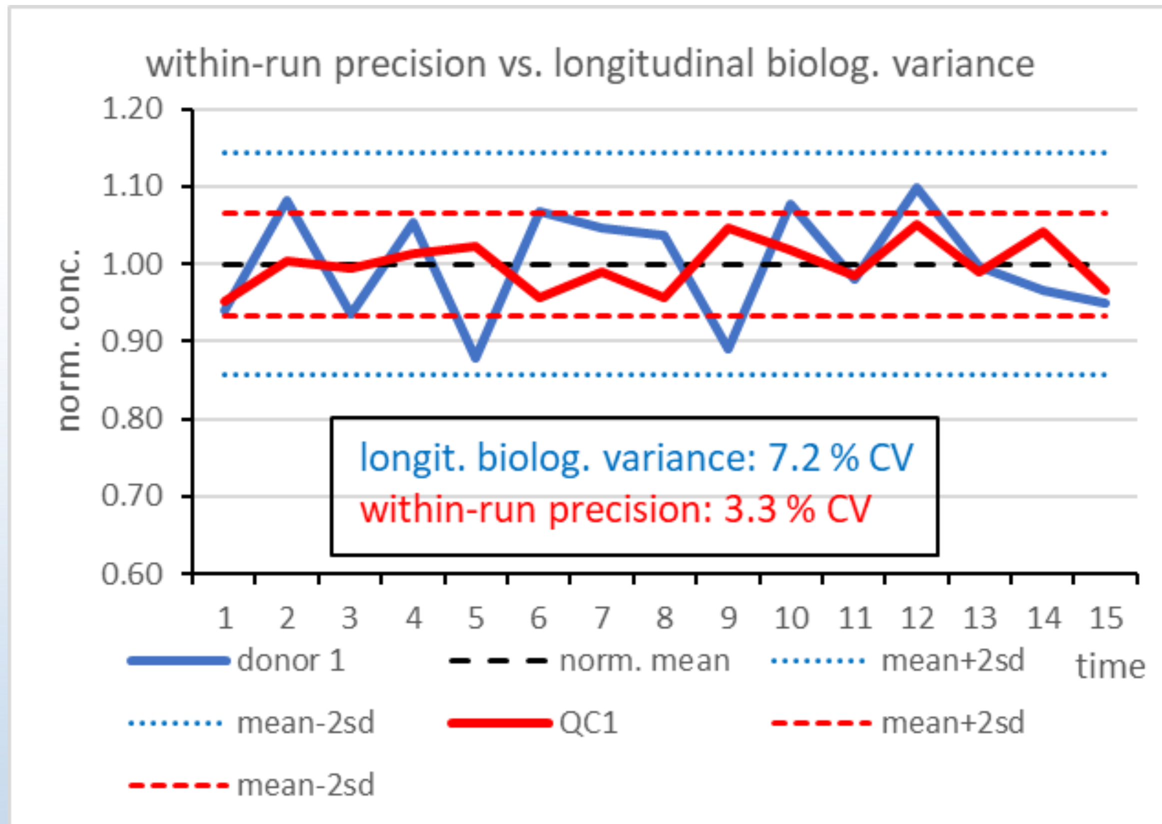
In total 15 validation runs on 8 days

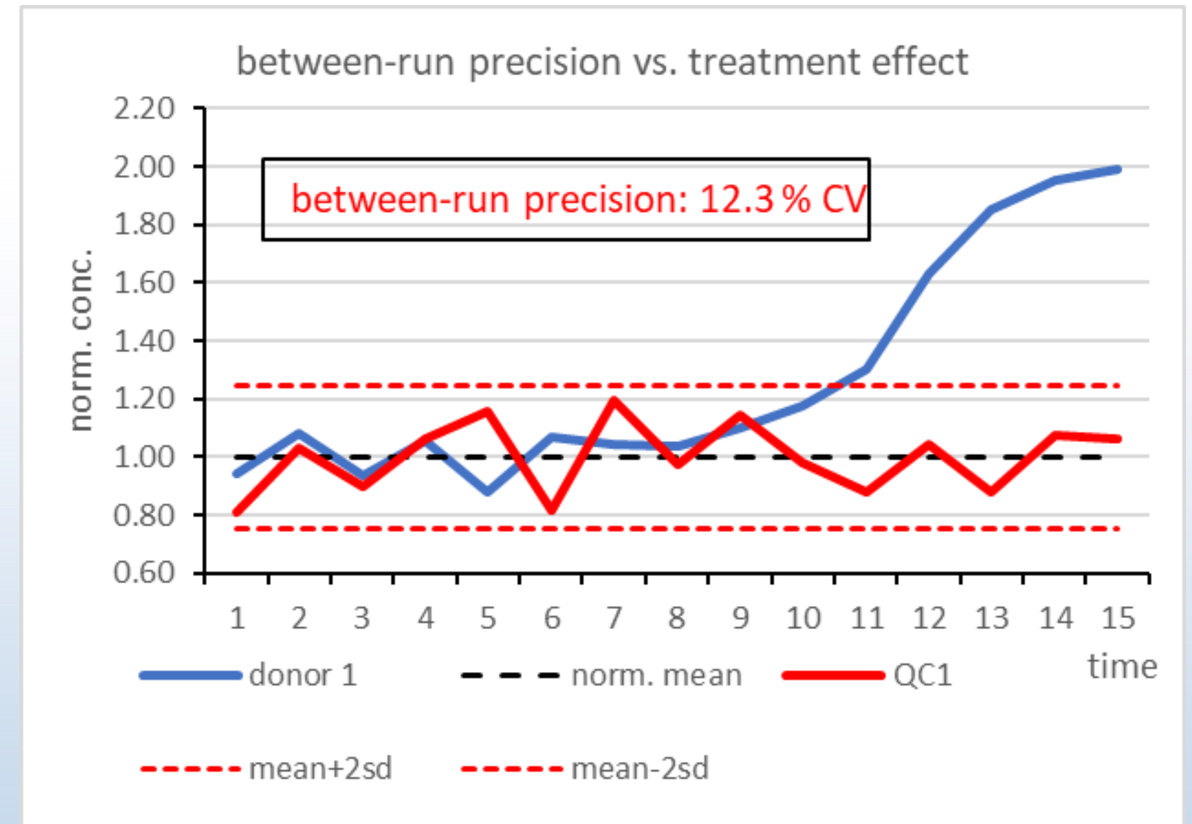
6. Validation plan (bioanalyst)

Validation Parameter	Experiment
Precision (within-run and between-run)	• 4 validation samples (plasma, diluted near LLOQ, low, mid, high range), four days and six runs and in three independent dilutions **at least**
Selectivity	• **Hemolytic plasma**: HV Plasma pure and spiked with 0.5% and 2% frozen/thawed/centrifuged whole blood of the same donor • **Lipemic plasma**: HV Plasma pure and spiked with 2% intralipid 20% (= equivalent to 400 mg/dL triglyceride), one aliquot after vortexing and one aliquot from the aqueous phase after centrifugation
Biol. variability intra-subject	• Banked longitudinal sample sets from at least three different donors
Biological variability inter-subject (healthy range)	• about 30 individual results • **Difference EDTA plasma, serum**: corresponding serum aliquots
Parallelism	• At least 6 individual samples from healthy volunteers **and patients (post-dose samples when available)** Samples should cover the whole range of possible dilutions (overlapping if possible, > two logs of dilutions).
Specificity	• **Interference of drug, interference of sTie-2**
Robustness / ruggedness	• **2 lab scientists** prepare calibration samples independently from each other. two runs
Long term stability stock solut.	• Ongoing monitoring project
Long term stability of endogenous analyte in matrix	• **Isochronic stability biobanking samples (5a, -20°C, -70°C + ref -150°C)**
Sampling stability	• stressed stability samples (**1hour at RT, 2 hours at 4°C) and 2000g and 4000g**, together with one immediately frozen reference plasma aliquot in one run • Three donors
Freeze Thaw stability fresh blood	• **fresh plasma aliquot**, split into two aliquots. One is analyzed immediately on the same day (Ref); other aliquots frozen at -70°C until measurement • Measurement in independent duplicates
Short term stability samples	• + 1F/T, +3 F/T at -20°C and -70°C, about 20 h RT
Stability calibration samples	• prepare **calibration samples twice**, store one set for about two hours at RT and analyze together with freshly prepared set

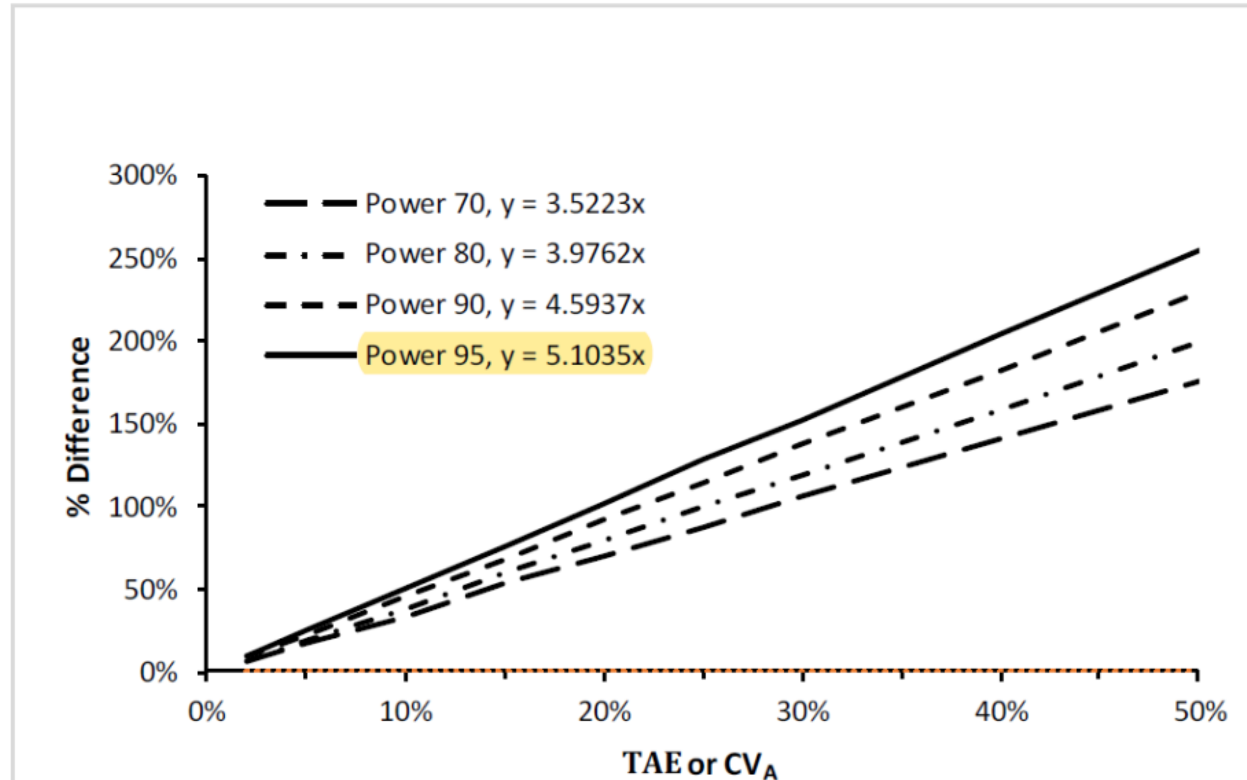
Choosing the acceptance criteria for the method - precision

- Within-run precision ≤ longitudinal biological variance (> not possible!)
- Between-run precision ??? (significant change after treatment)





Longitudinal biological variance and analytical precision have an influence on significant change detection



Piccoli et al. Points to consider. C-path Institute, 2019

- For example: A two fold increase of BM level (100%) require a between-run CVA < 20% (Power 95)
- Maximum between-run precision acceptance criterion

Choosing the acceptance criteria for the method

Validation Parameter	Acceptance criteria for method
Precision (**between-run**)	**< 20% CV** IF precision ≥ longitudinal biological variance (= enables detection of two fold increase), ELSE < longitudinal biological variance
LLOQ / ULOQ	**LLOQ < healthy volunteer level**
Healthy volunteer level	**None**, just report
Parallelism	**Yes in 5 out of 6 individual subject samples**, dilution range > 2 logs
Specificity (drug tolerance)	Rel. Dev. from unspiked sample < 20% up to Cmax of highest planned dose group
Selectivity	No significant interference of lipemic samples or weak hemolytic
Stability calibration samples	*80% of deviations of calibrators <u>within three times of with-in run precision</u>*
Short term stability of endogenous analyte in matrix	*CVs of replicates < 25%, deviations from reference < three times of within -run precision*
Long term stability of endogenous analyte in matrix	*80% of deviations vs. reference within three times of within-run precision, no trend with time or temperature*

Summary of validation results

7. Assay **validation**
(bioanalyst + lab/CRO)

Validation Parameter	Result
Precision (within-run and between-run)	Within-run: 1-4% CV (N=7x3) Between-run: 3-6% CV (N=3x7)
Selectivity	Hemolytic: no influence Lipemic: no influence (vortexing or centrifugation)
Biol. variability intra-subject (longitudinal)	**7% CV** (N=3x15, over 29 days) Very low = between-run precision
Biological variability inter-subject (healthy, = reference range)	1.1 – 4.8 ngeq/mL, **42% CV** (N=24) No significant difference Serum/Plasma (ratio 1.08)
Parallelism	Yes 1:2 – 1:20000, no trend (*even in patient post-dose samples*)
Specificity	No interference of drug and sTie-2
Robustness / ruggedness	Calibration curves from both lab scientists comparable
Long term stability stock solut.	Stable at -70°C for >1287 days (3.5a, *post-study results*)
Long term stability of endogenous analyte in matrix	Stable at -20 and -70°C for > 5a
Sampling stability	Ok for 1h RT, 2h cooled No difference 2000g vs. 4000g
Freeze Thaw stability fresh blood	ok
Short term stability samples	Stable + 1F/T, +3 F/T at -20°C and -70°C, about 20 h RT
Stability calibration samples	Stable for > 2h at RT

Analytical Work Plan

8. Analytical Work Plan (bioanalyst) + **Measurement of samples**

- Context of use of the BM measurement in this particular study
- Definition of Method (method description, version, incl. critical reagents, standard), link to validation project, possible deviations from method description incl. justification that these have no influence on the validity of the method,
- **Statement that the above method is suitable and sufficiently validated for the CoU in the study to obtain reliable results**
- ~~Measurement of all samples of a subject together in the same run = lowest imprecision and bias~~

Not possible due to frequent interim analysis!

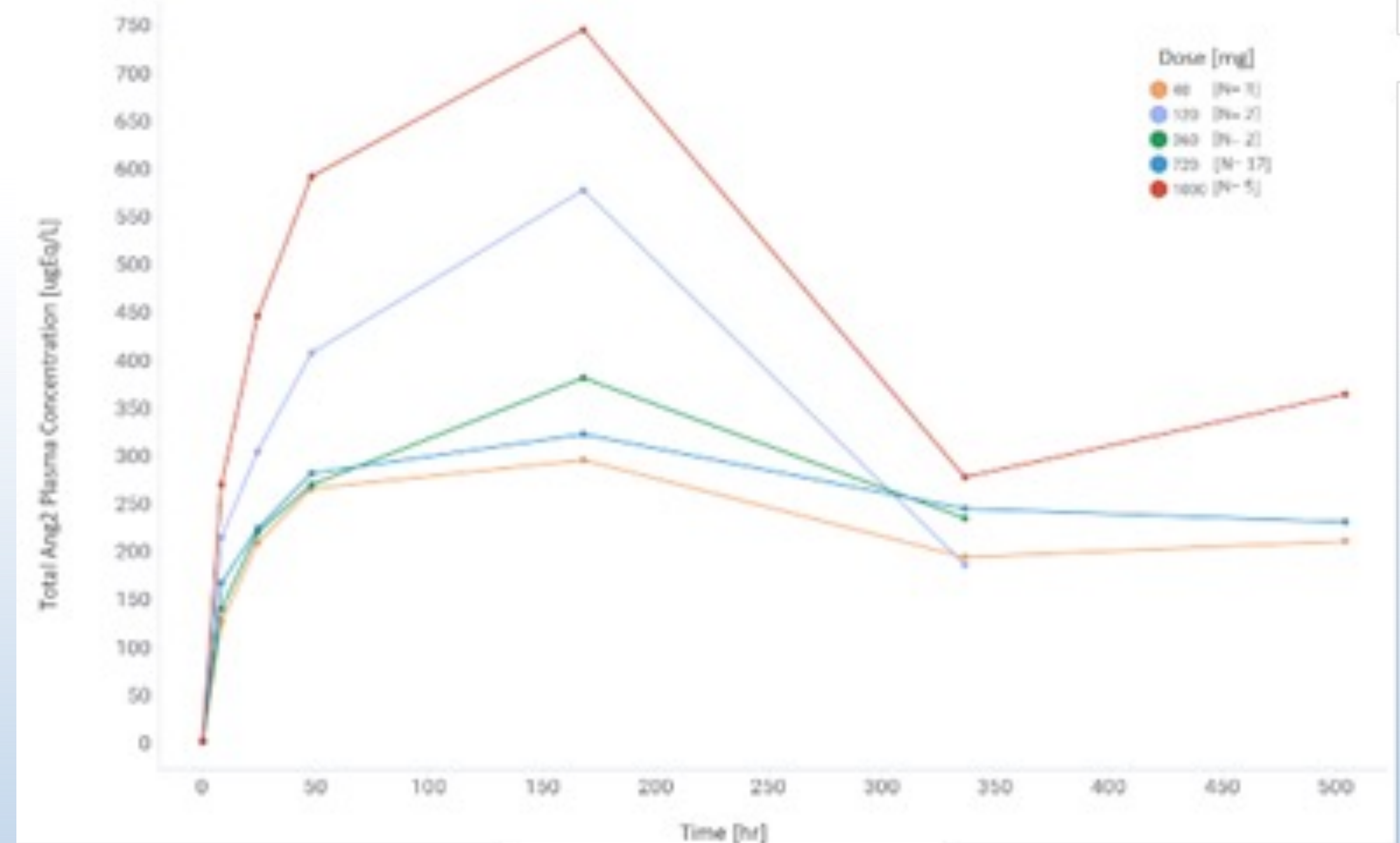
- Daily **QC run acceptance**, QC target level determined during validation or during bridging runs, rel. dev. from target = 3 times between-run precision (20 %)*

* Changed during study,

- **Further parallelism** testing (6 patient samples with high concentration) as soon as available
- **Assay suitability runs** if assay not used for longer than 6 month (confirmation of reagent stability)
- **Bridging runs** of new stock solutions (reconstitution of lyophilized standard), reagents (e.g. new labeling)
- **Partial revalidation** in case of new lots of critial reagents (parallelism and HV range N=30), min every second year

Outcome – trial results

- > 100 fold increase in total Ang-2 level after treatment (much more than expected, lack of info from modelling)
- Some problems with outlying runs due to QC deviation > 20%
  - Increase of QC acceptance criterion to 30%, sufficient due to observed BM change
- Clinical study measured over three years with comparable results
- Assay still in use for further studies



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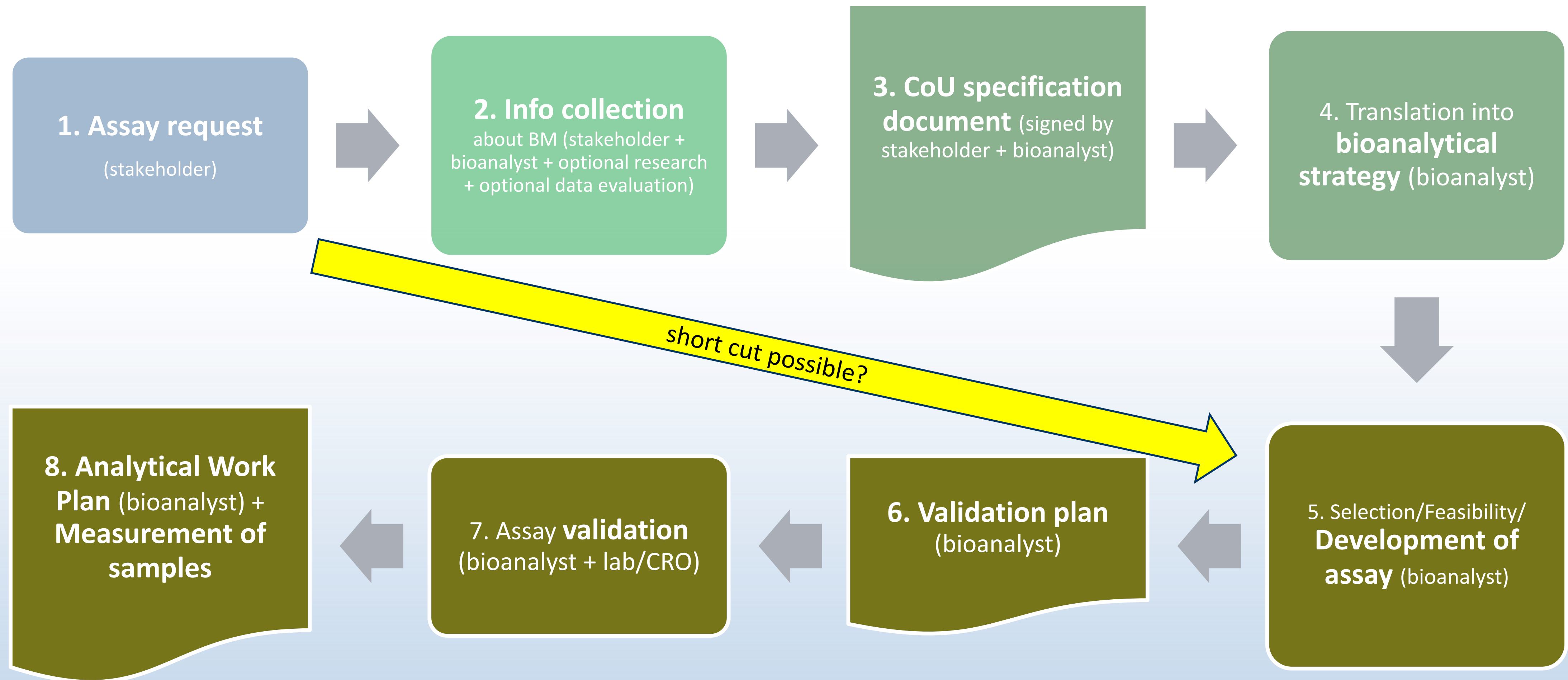

What would have happened without the prospective CoU definition?

Missing information about	Possible consequence in assay validation	Possible consequences on data interpretation
required specificity (e.g. **total** target)	Commercial kit instead of homebrew assay (not total, not free but something in between)	Misleading data interpretation (failed pk/pd model verification
Required sensitivity	Wrong assay range	Below lower limit of quantification results only
Data evaluation	Maybe a quasi-quantitative assay would have been accepted	No valid concentration data, not useful for modelling.
Frequent need for interim data	Storage of insufficient aliquots of reagents and QCs	Frequent bridging of lots may cause additional bias on data
Treatment effect on BM	Wrong assay range, insufficient precision	Treatment and biological effect masked by analytical error
Duration of trial, need for long term comparability of data	Lack of stability information, insufficient method robustness	Additional bias on data, risk of non-comparable results or even not valid results

Questions?