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EBF Spring Focus Workshop 2024

IVDR: our next Challenge?

NH Malaga, Malaga, Spain

Proximity Extension Assay (PEA) as novel technology for pharmacodynamic assessments and CDx?

Toralf Roch – 06th June 2024

PK determination of receptor binding immuno-suppressive drugs could be challenging

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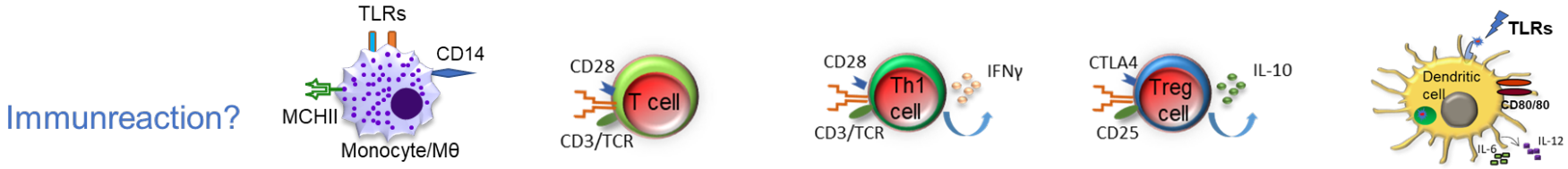
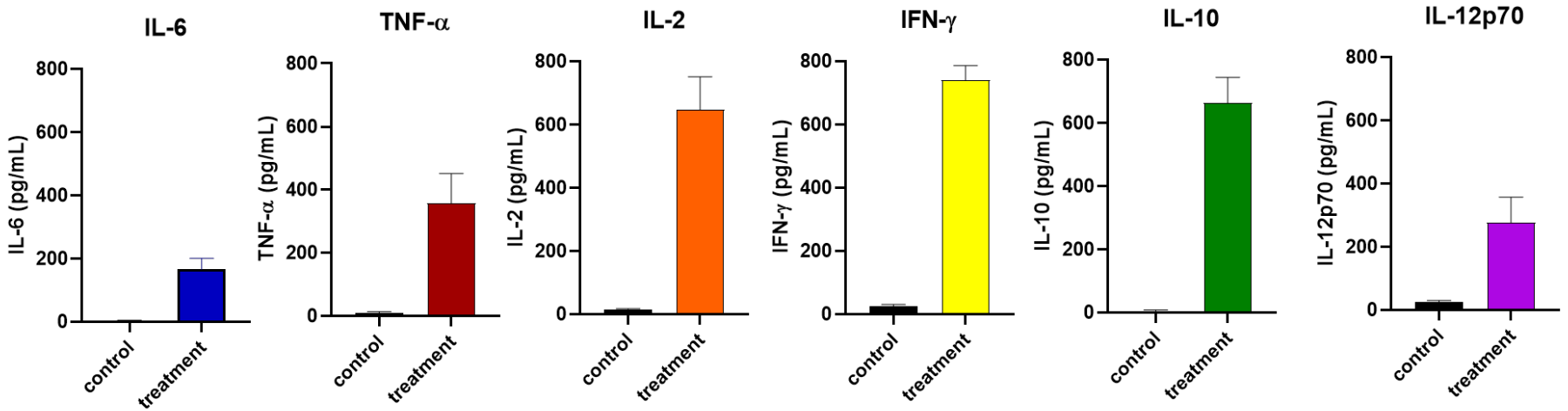
- PK assessment of antibody- and other protein-based drug are challenging to quantify due to efficient target binding
- PD analysis in early phase may reveal drug efficacy
- Comprehensive immunological assessments require the analysis of multiple factors





In vitro assays are necessary to analyse inflammatory responses – example from a clinical study

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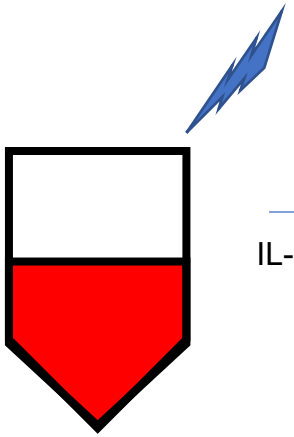
- In healthy individuals the anti-inflammatory drug effects cannot be observed



Inflammatory trigger → mimicking an inflammatory response

→ IL-6, TNFα, IL-8

Readout using a validated 3-plex

↓

Validation of the Stimulation was performed (intra-, inter-, and AoB) → LDT (IVDR conform?) → Ranges of cytokines production not available and difficult to obtain → high and low responder

↓

Inhibitory drug effects can be observed

Contradictory sponsor Statements:
“Validate the stimulation assay!”
“Validation of stimulatory assays is not possible, therefore not required…”

Can such a rather complex setting be IVDR conform?

Proximity extension assay (PEA) determining the expression of 92 analytes as readout for the stimulation assay

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→ Establish the proximity extension assay (PEA) allowing to detect 92 inflammatory mediators instead of using the validated MSD 3-plex

→ Considering/labelling this assay as “exploratory only” may not be sufficient, when the data are used as PD marker and potentially safety considerations

→ Is validation of the PEA an option considering common issues with multiplex assay validations?

Our validation experience from MSD multiplexing:

- High complexity in analysing multiple calibration curves and QCs
- Dynamic Range Limitations (Samples Dilutions)
- Cross-Reactivity
- Matrix Effects
- Instrument/Software complexity

How would this translate to an assay with 92 analytes that “only“ determines relative differences and for which data normalization is crucial?

The Proximity Extension Assay (PEA) technology

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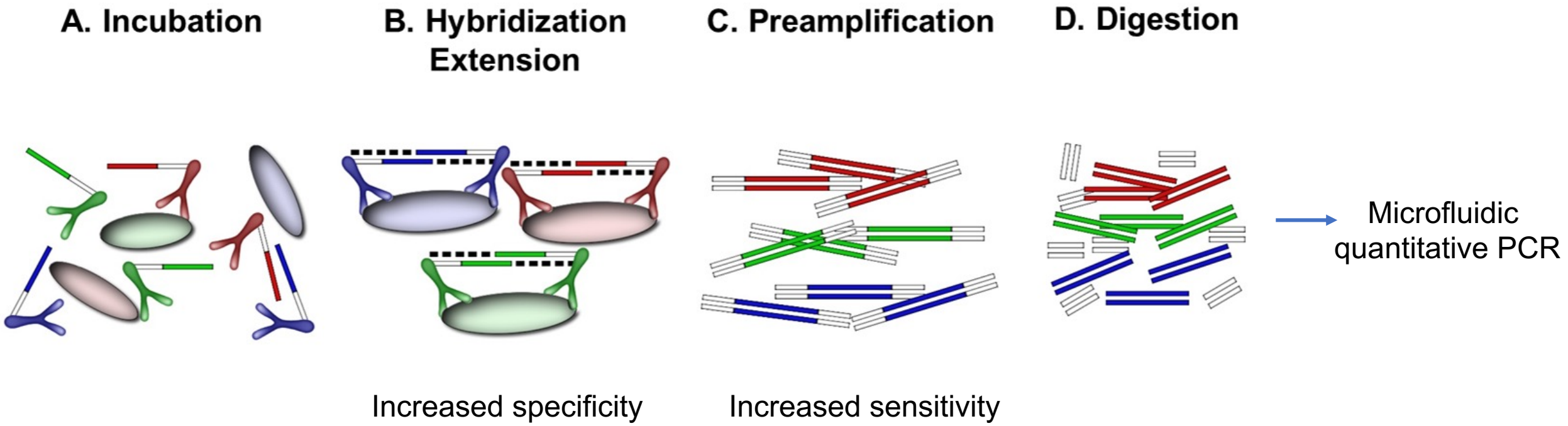

Assarsson E. et al. (2014) Homogenous 96-Plex PEA Immunoassay Exhibiting High Sensitivity. Specificity. and Excellent Scalability. PLoS ONE 9(4): e95192. https://doi.org/10.1371/journal.pone.0095192

Microfluidic qPCR generates Ct values as raw data

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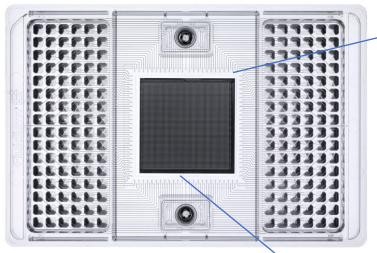
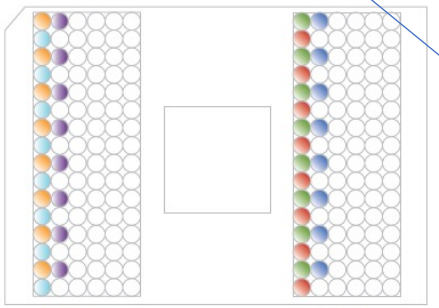
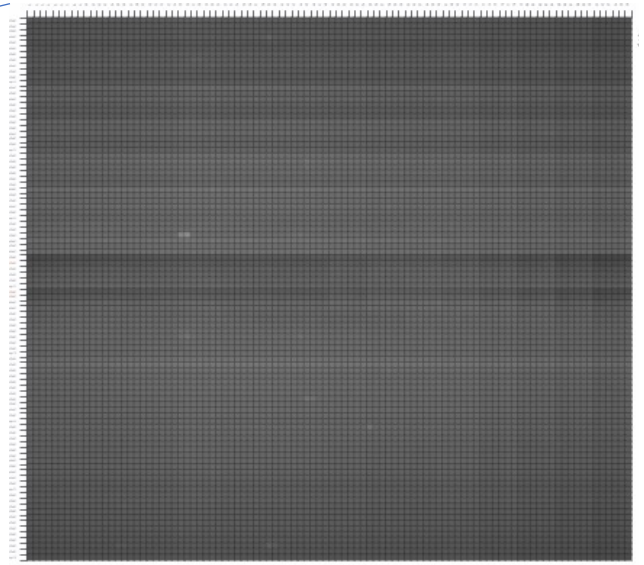
96.96 Integrated Fluidic Circuits (IFC)





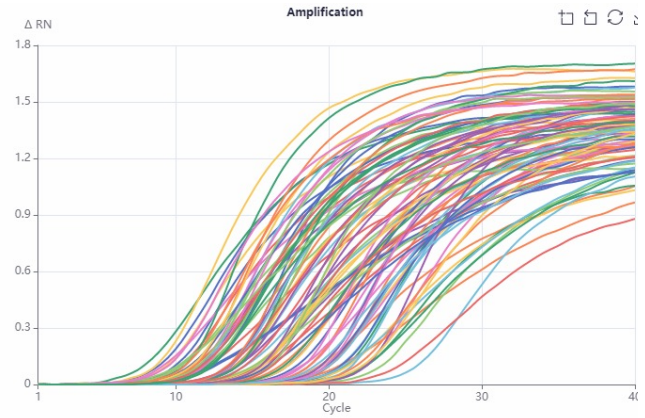
Primers Samples

9216 qPCR reactions on one chip



Signature Q100

Amplification curves of 92 analytes and 4 controls for one sample



Ct values as raw data

The PEA has build-in Quality Controls (QC), which are used for the run validity evaluation

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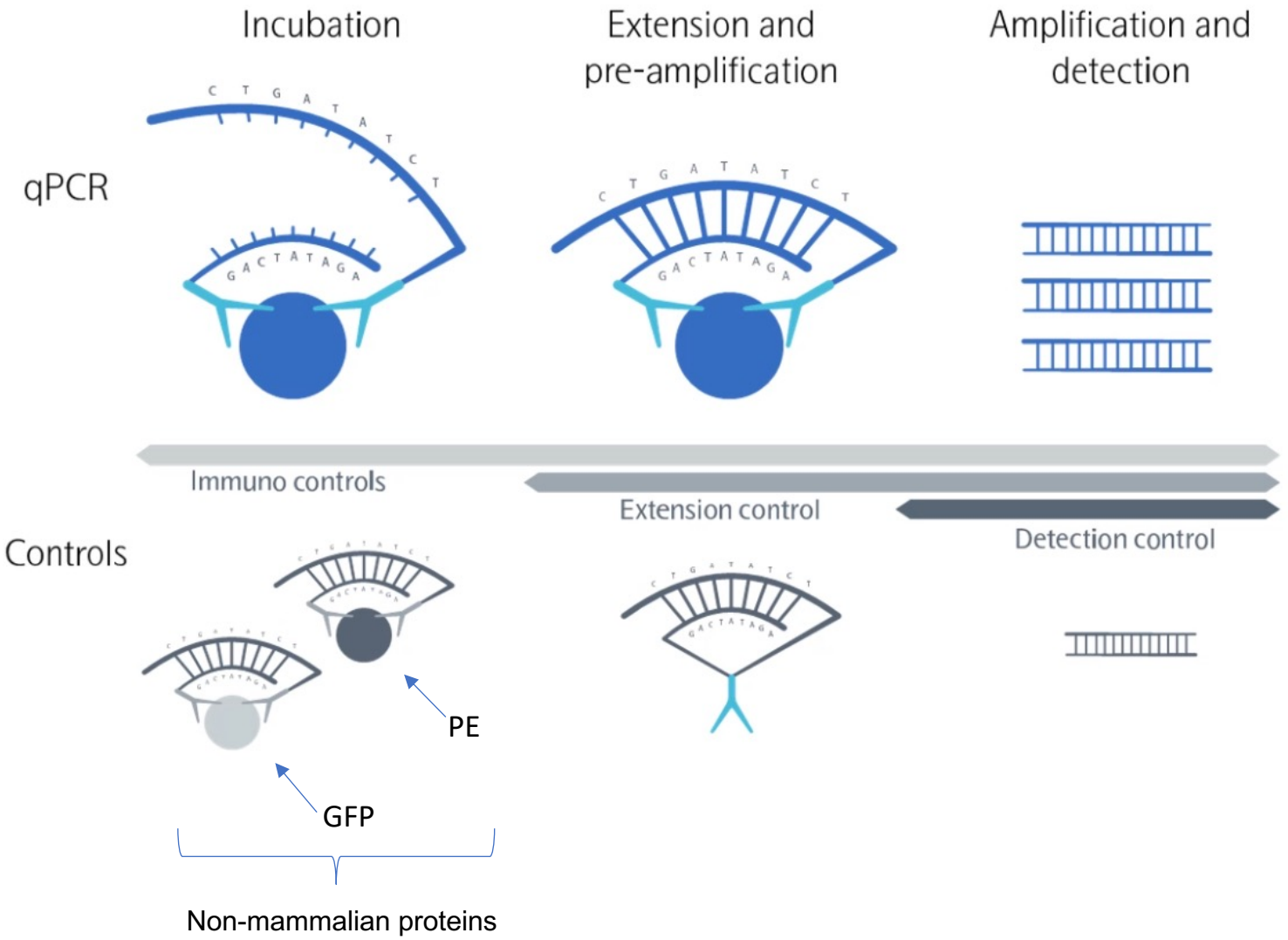

Calculating Normalized Protein Expression (NPX) values

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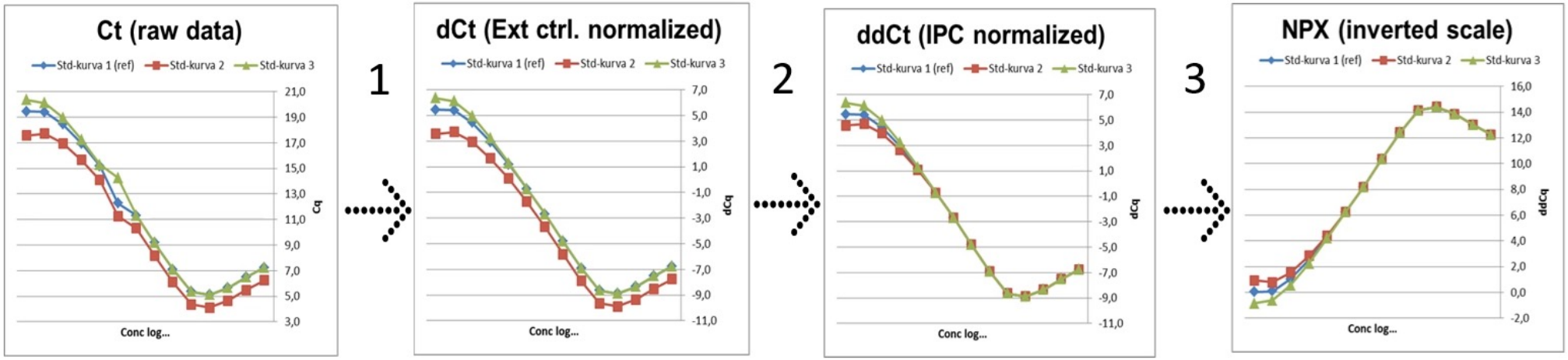
1. $Ct_{(analyte)} - Ct_{(Extension\ control)} = dCt_{(analyte)}$ — Decreases technical „well to well“ variation
2. $dCt_{(analyte)} - dCt_{(median\ IPC)} = ddCt_{(analyte)}$ — Considers variation between plates
3. $Corr.\ factor_{(analyte)} - ddCt_{(analyte)} = NPX_{(analyte)}$ — For more intuitive data representation



- NPX is a relative unit
- Normally distributed on a log 2 scale
- High NPX = High protein expression

https://www.olink.com/content/uploads/2021/09/olink-data-normalization-white-paper-v2.0.pdf

Run evaluation to identify technical variations

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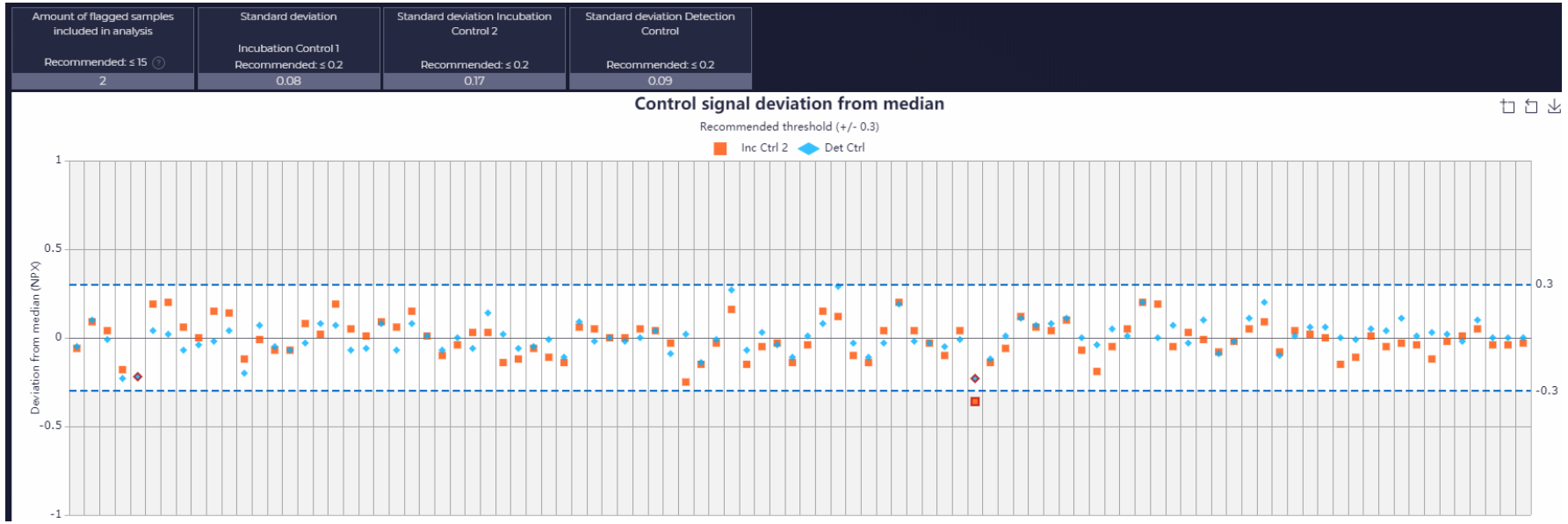
- Incubation and detection control are in each samples and should have similar NPX values varying below +/- 0.3.
- Increased deviation from of the median from the internal incubation and detection controls results in sample flagging



Reasons for increased flagging:

- Matrix (assay optimized for EDTA plasma)
- Dilution factor and diluent
- Activation or disease status
- Air bubbles

→ Flagged samples should not be considered as failed

→ Increased flagging requires a details technical evaluation of the run

The complex setting used in a clincial study

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- Validated stimulation *in vitro* assay w/o defined ranges
- High donor to donor variance
- Multiplex readout with relative units (NPX) for which different normalization methods can and have to be applied

What we did:

- Assay comparison MSD vs PEA
- Dilution testing
- Intra assay variance determination
- Inter assay variance determination
- Comparing normalization methods

Comparison of MesoScale multiplexing immunoassays vs PEA in a LDT

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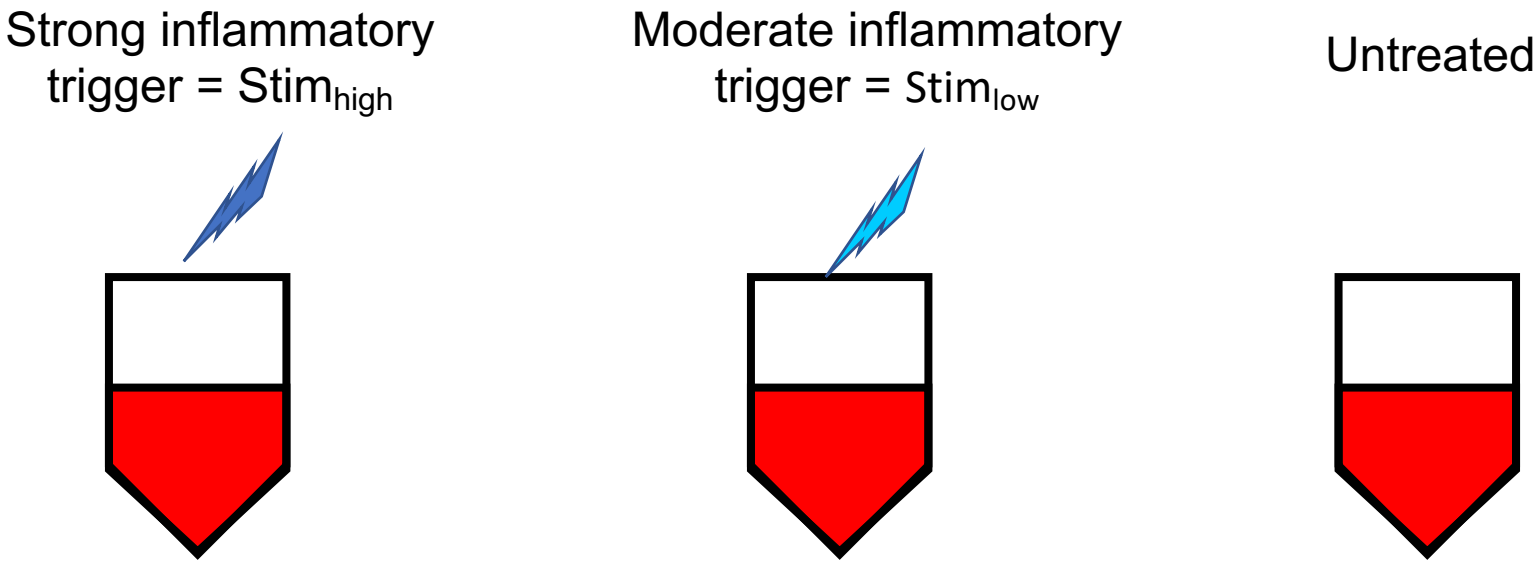

- Incubated for 20 hours and supernatants stored at -80˚C for at least 24 hours
- MSD and PEA performed triplicates from three different donors (low, intermediate, and high cytokine producers)

High donor variance is observed in both assays

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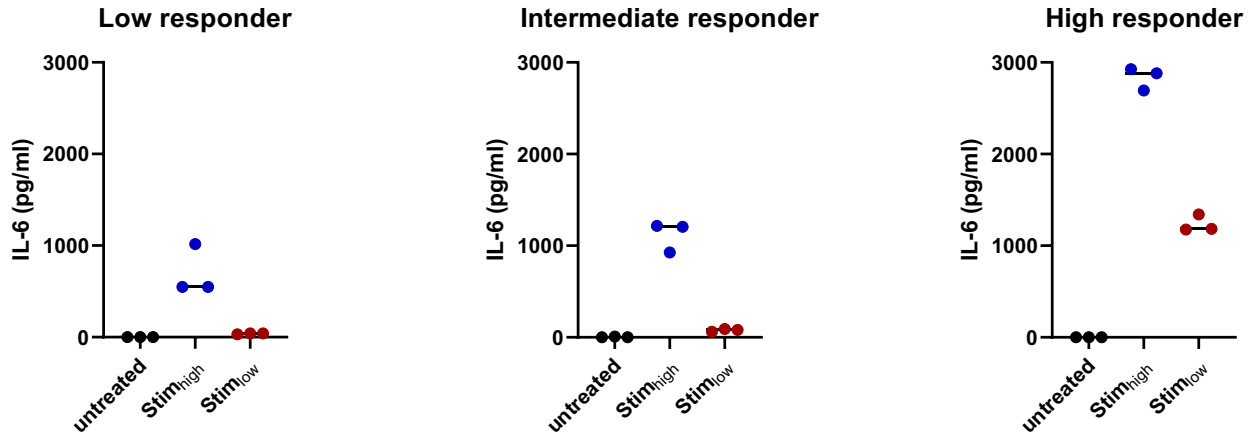
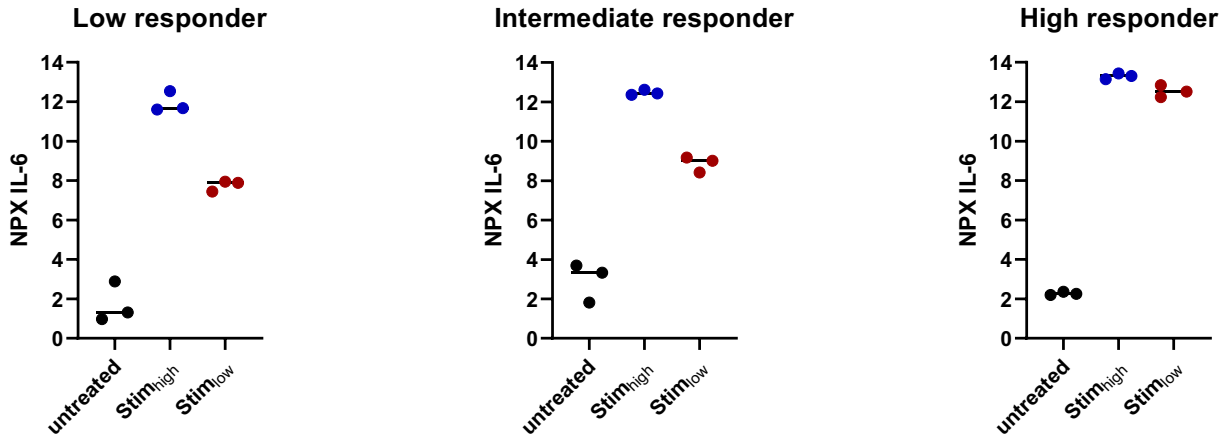
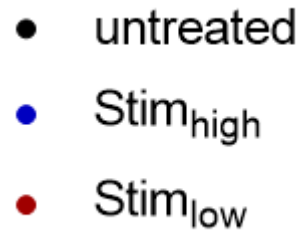
MSD



PEA





Similar data were obtained for IL-8 and TNFα

Interestingly, the PEA give values in samples, which are below detection limit in MSD

Finding the optimal dilution factor for the PEA assay is challenging

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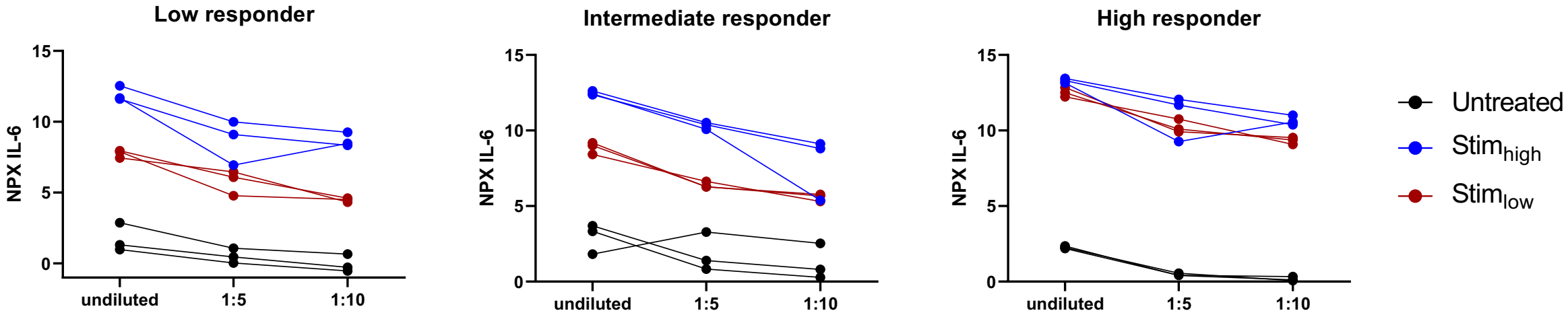


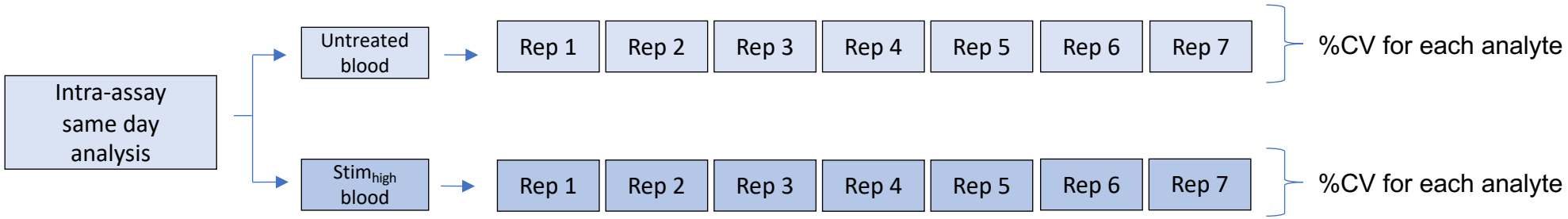
Differences may disappear when analytes are expressed at very high or low level

Intra-assay validation experiments

Validation samples:

- Pooled samples from untreated and $Stim_{high}$ blood

Intra assay variance



Intra assay variance calculation example – PEA assay

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Representative %CV calculation for six analytes

	IL8	VEGFA	CD8A	MCP-3	IL-6	TNF-α
NPX	10.6	10.4	6.8	4.3	2.57	3.26
	11.0	10.7	6.9	4.5	2.81	3.41
	10.9	10.6	6.8	4.6	2.80	3.33
	11.0	10.6	6.9	4.7	2.83	3.55
	10.9	10.6	6.9	4.5	2.76	3.48
	10.9	10.4	6.8	4.5	2.67	3.12
	10.8	10.6	6.8	4.4	2.97	3.40
2^{NPX} (linearized NPX)	1529.7	1360.9	107.8	20.2	5.94	9.58
	2040.9	1623.9	120.8	22.8	7.01	10.64
	1871.5	1548.6	114.0	24.4	6.94	10.08
	2008.0	1587.1	119.5	26.8	7.09	11.71
	1860.2	1538.0	117.6	22.0	6.78	11.16
	1849.6	1394.1	111.8	22.6	6.37	8.67
	1729.6	1588.2	113.0	21.4	7.81	10.54
Standard Deviation 2^{NPX}	172.5	101.9	4.6	2.1	0.59	1.01
Mean 2^{NPX}	1841.4	1520.1	114.9	22.9	6.85	10.34
%CV 2^{NPX}	**9.4**	**6.7**	**4.0**	**9.3**	**8.59**	**9.74**

- For the %CV calculation the log NPX values have to be linearised (2^NPX)

Intra assay variance $Stim_{high}$ sample– PEA assay

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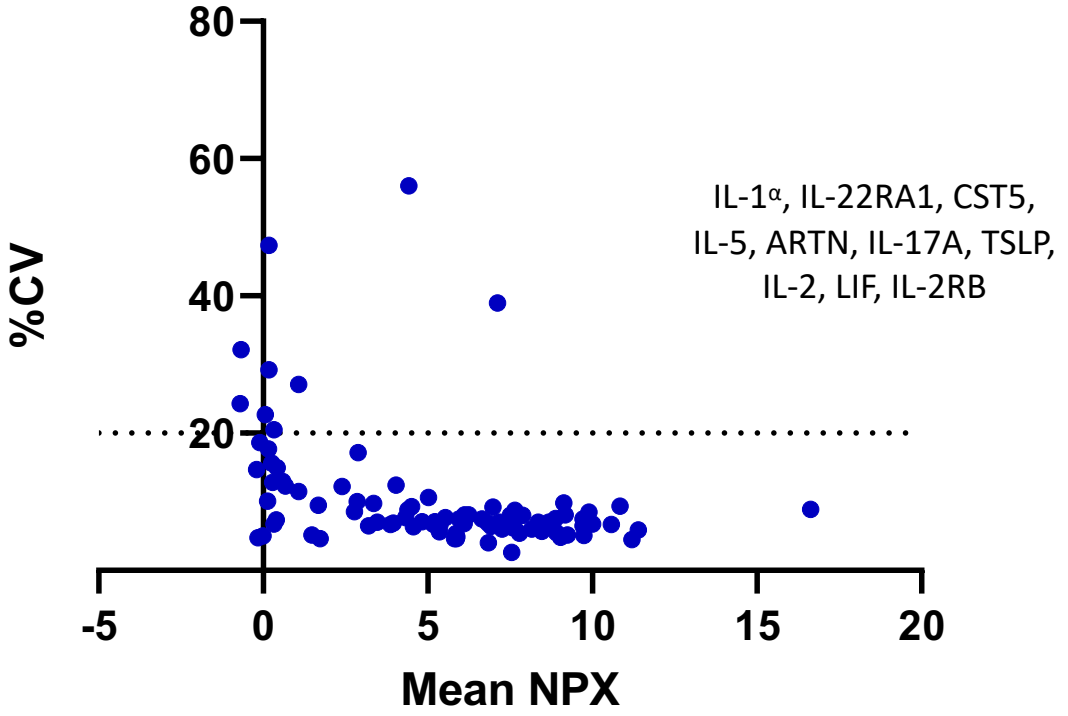
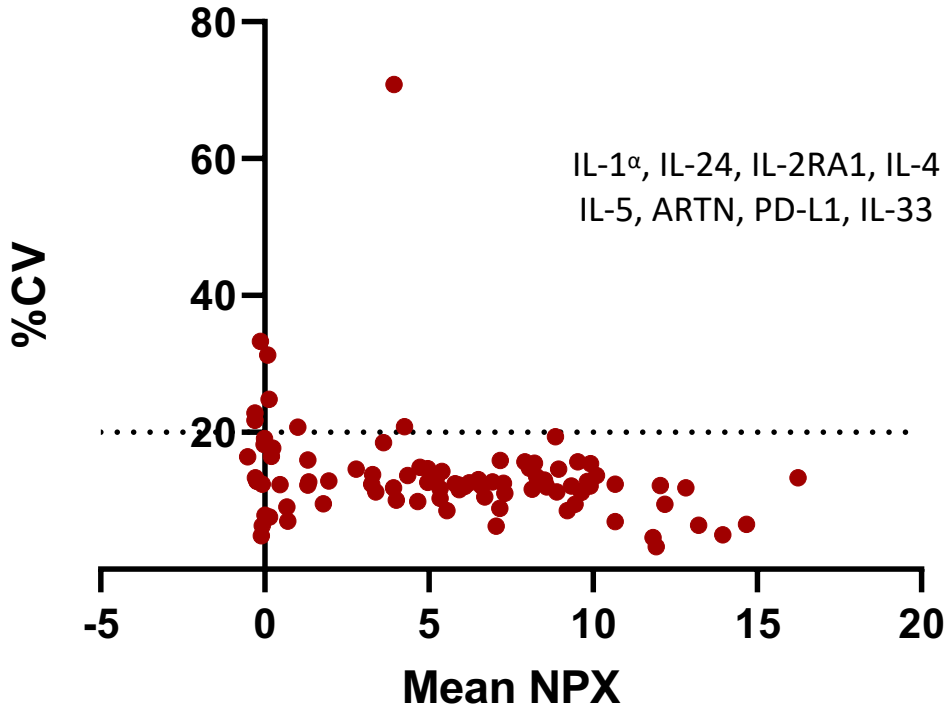
Correlation of %CV and NPX values for untreated blood



Correlation of %CV and NPX values for $Stim_{high}$ blood



Considering both conditions 15 out of 92 (16.3 %) analytes showed a intra assay %CV above 20

There will always be analytes that do not fulfil the acceptance criteria

Inter-assay validation experiments

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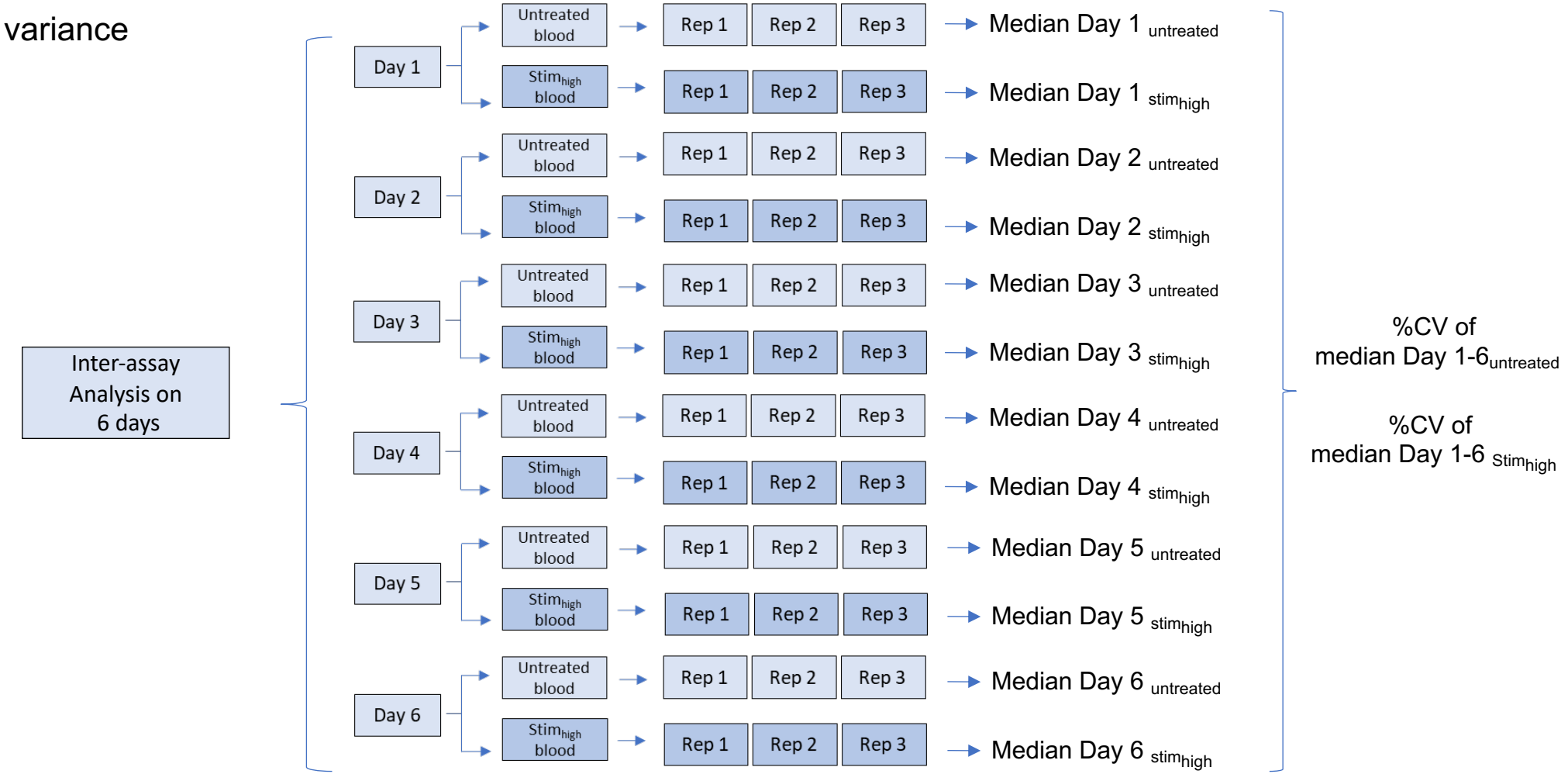
Validation samples:

- Pooled samples from untreated blood and $Stim_{high}$ blood

Inter assay variance



Randomized study samples → **intensity normalization**

- Increase statistical power and reduce technical variation between plates

Non-randomized study samples → **reference sample normalization**

- Running reference (bridging samples) on all plates to minimize technical variation

white-paper-data-normalization-v2.1.pdf (olink.com)

Intensity normalization method

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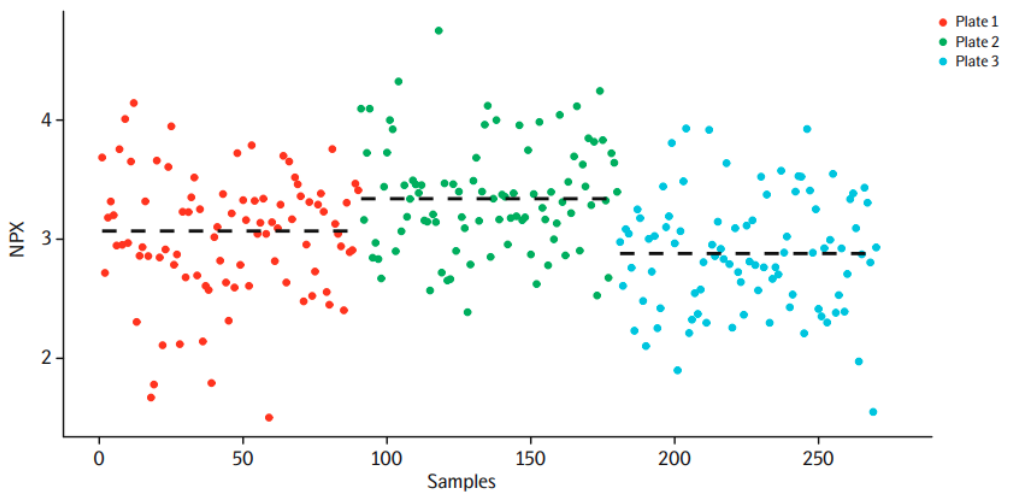
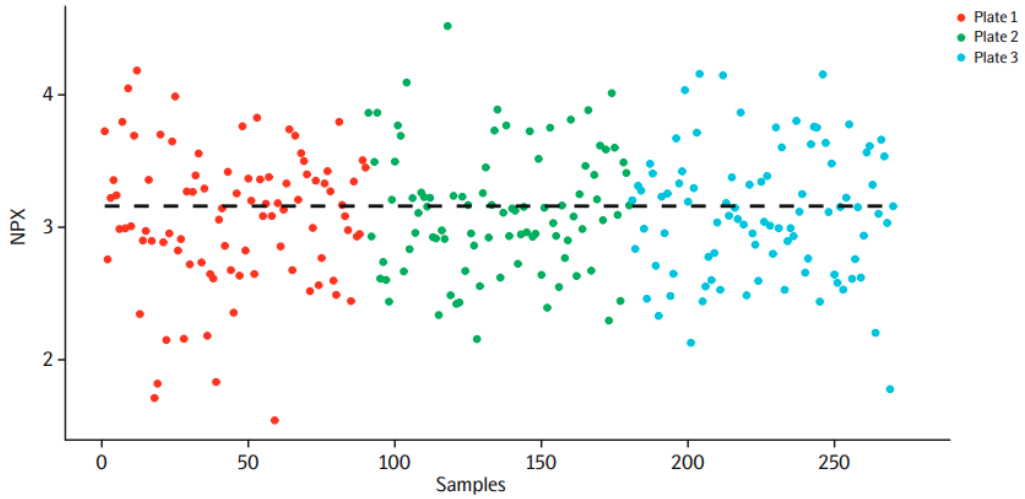
Assumption:

- Well randomized samples → no expected difference between the median signal for an assay on one plate compared to another
- If any such difference is observed between plates, it can be interpreted as technical bias and be safely removed. resulting in more comparable values

Before intensity normalization



After intensity normalization



- normalization performed for each analyte individually

white-paper-data-normalization-v2.1.pdf (olink.com)

Reference sample normalization

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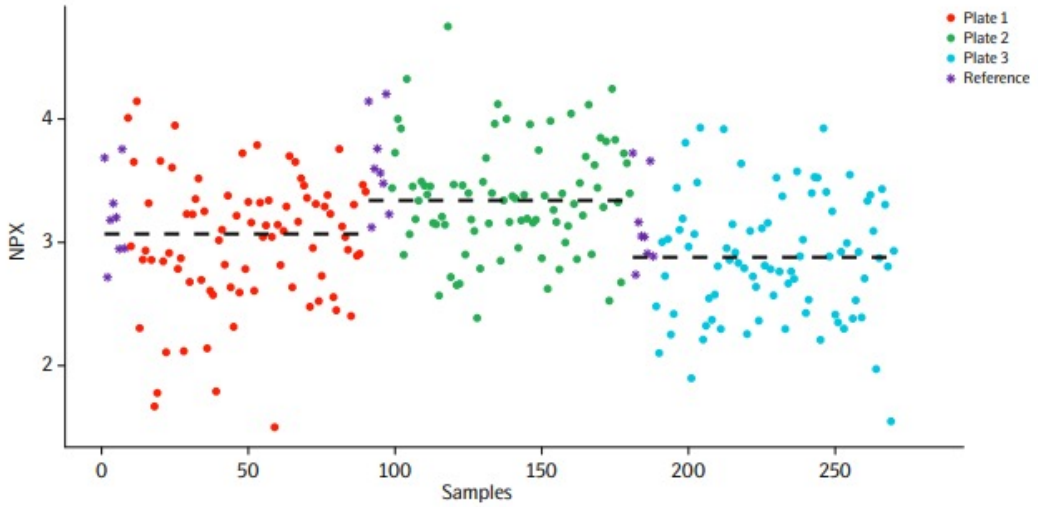
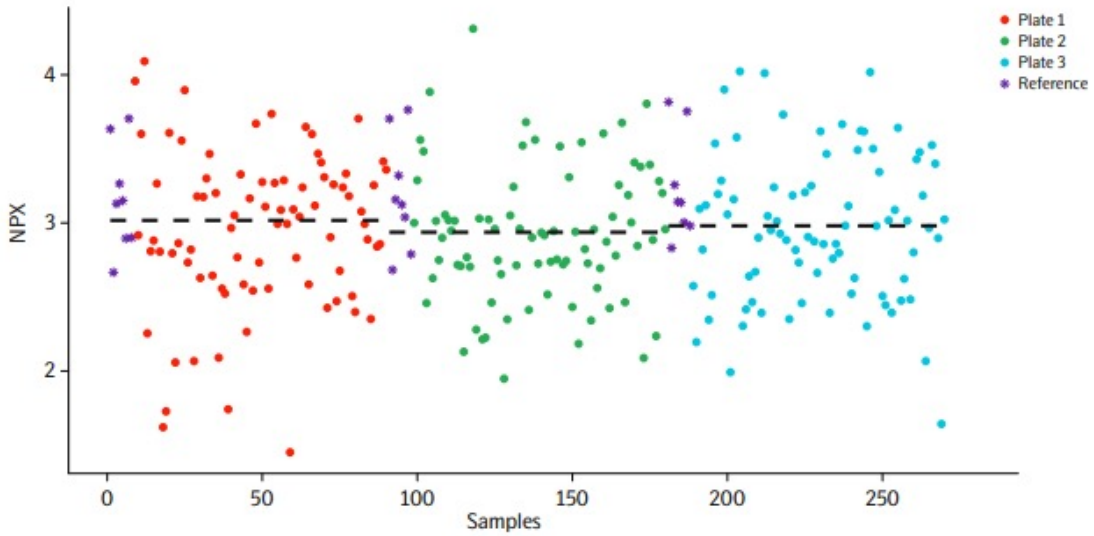
Assumption:

- samples are poorly randomized
  - → technical variation between plates is mixed with the biological variation
  - → intensity normalization of the plates would remove some of the relevant biological variation
- After reference sample normalization, the median NPX can still differ between plates, reflecting different compositions of sample groups

Before reference sample normalization



After reference sample normalization



- normalization performed for each analyte individually

white-paper-data-normalization-v2.1.pdf (olink.com)

Inter assay variance of untreated blood

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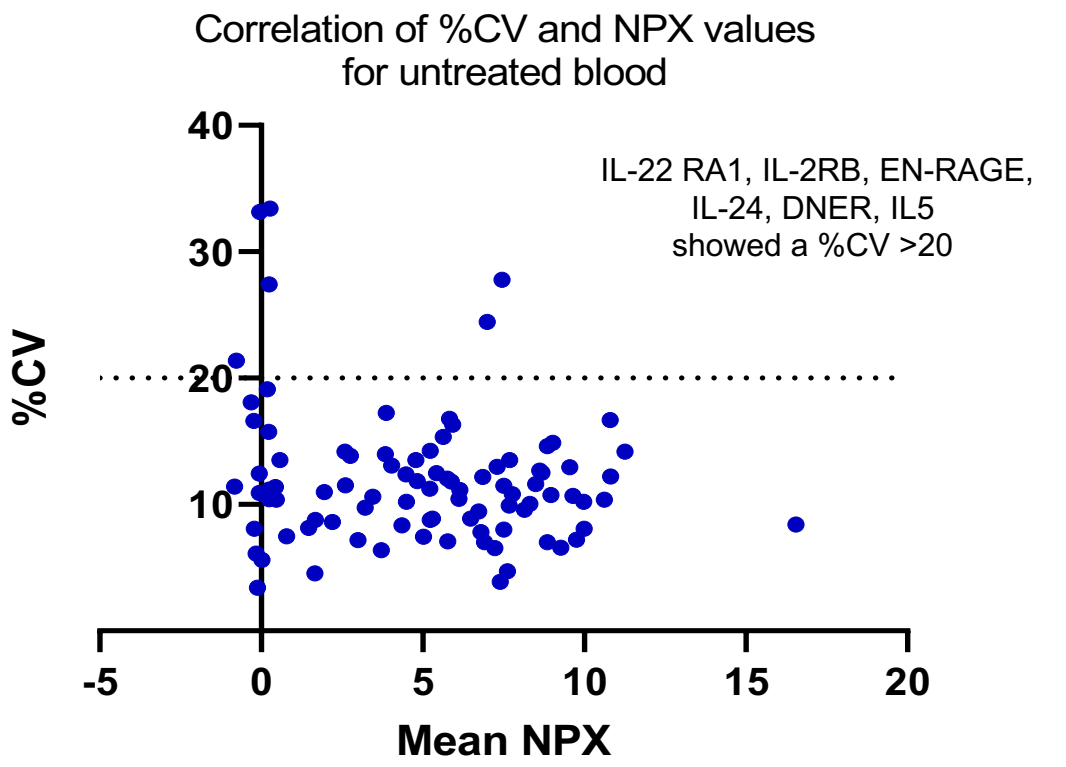
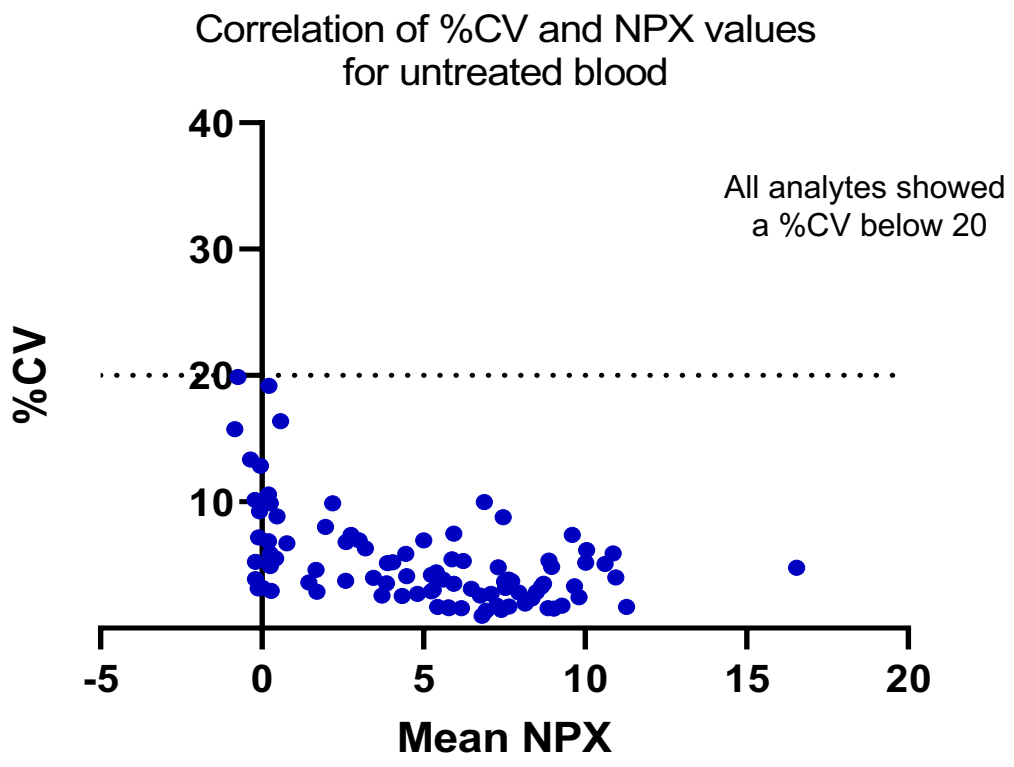
No normalization between plates



Intensity normalization



For each analyte the mean NPX of 6 independent analysis days is plotted against the %CV

%CV calculated with linearized NPX

Inter assay variance $Stim_{high}$ sample

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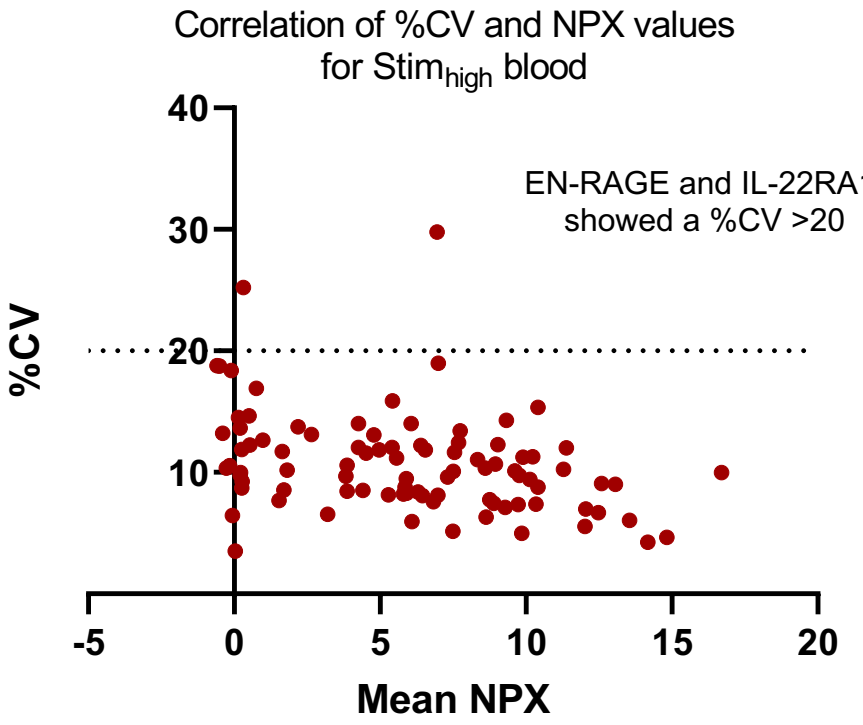
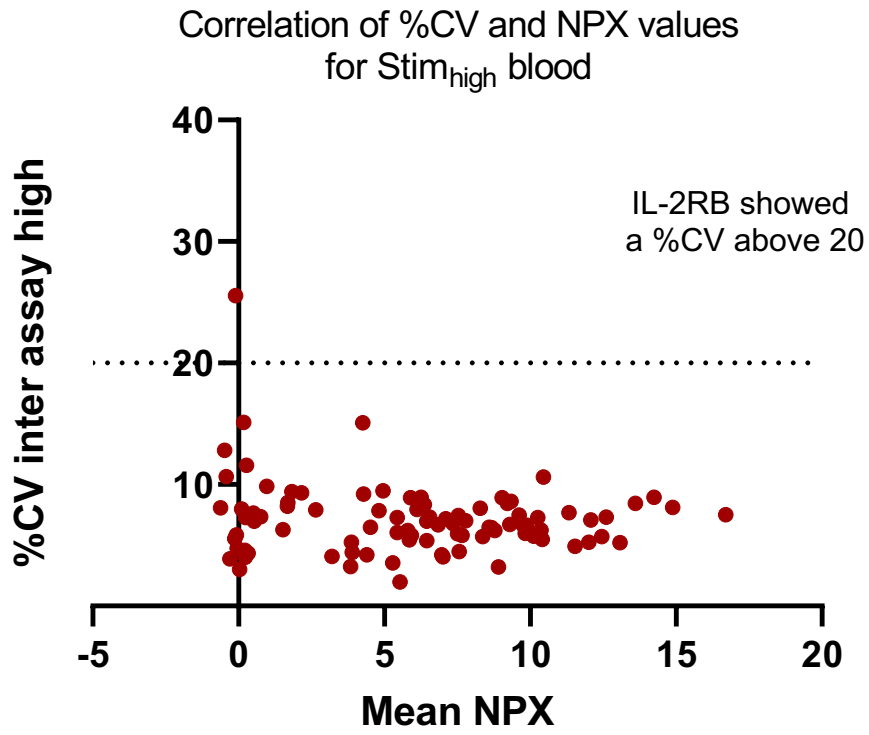
No normalization between plates



Intensity normalization



What kind of variance (normalization) would be considered for the assay validation and data reporting?

We provided data to the sponsor that were normalized in different ways → risk of cherry picking!

%CV calculated with linearized NPX

Conclusion and overall experience with the PEA system

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- The PEA provides comparable data as the MSD system with a similar high sensitivity
- Sample dilution is possible and might be relevant for a certain study samples
- Intra- and inter-assay variance is for most analytes below 20%CV
- Integrated quality controls and intuitive analysis software

- Cost-, material-, and labour-intensive
- Intense hands-on training required, which is guided by an excellent technical support
- Run validity assessment is challenging and requires intensive training
- Complex Data and normalization methods
- Service provider and sponsor need bioinformatical expertise
- Frequent software updates
- Implementation of a 21 CFR Part 11 analysis software module

Discussion

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- Complex setting that include LDT combined with multiplex analysis are challenging in light of IVDR
- Sponsors ask for such approach at least in Phase I and in FIH studies
- To which extent the IVDR can really be applied to multiplex assays remains to be discussed

Acknowledgment

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Contact us

CheckImmune GmbH
Campus Virchow Klinikum
Cranach Haus
Augustenburger Platz 1
13353 Berlin

web: www.CheckImmune.com

email: andreas.hueser@checkimmune.com
toralf.roch@checkimmune.com

phone: +49 30 450524062