Highly sensitive Cytokine Quantification using Imperacer®

an Immuno-PCR based Ligand-Binding Assay System

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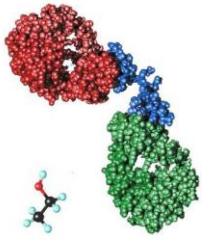
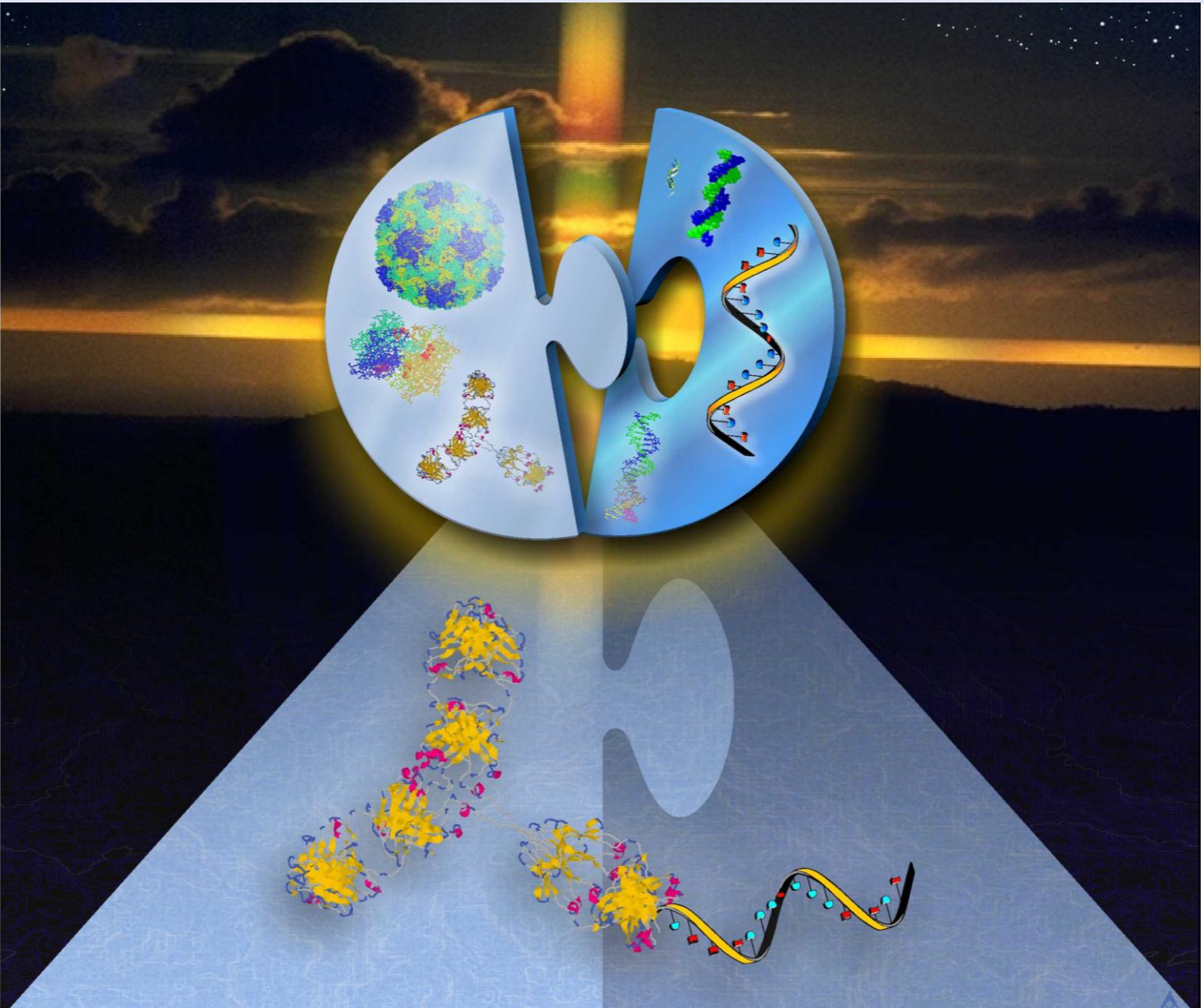
EBF Focus Meeting „*Large meets Small*“ 2011



Ultrasensitive Target Detection with New Analytic Tools



Functional elements form nature‘s „*Biomolecular toolbox*“ are combined to novel applications

Recognition

Coupling

Structure

Signal Generation

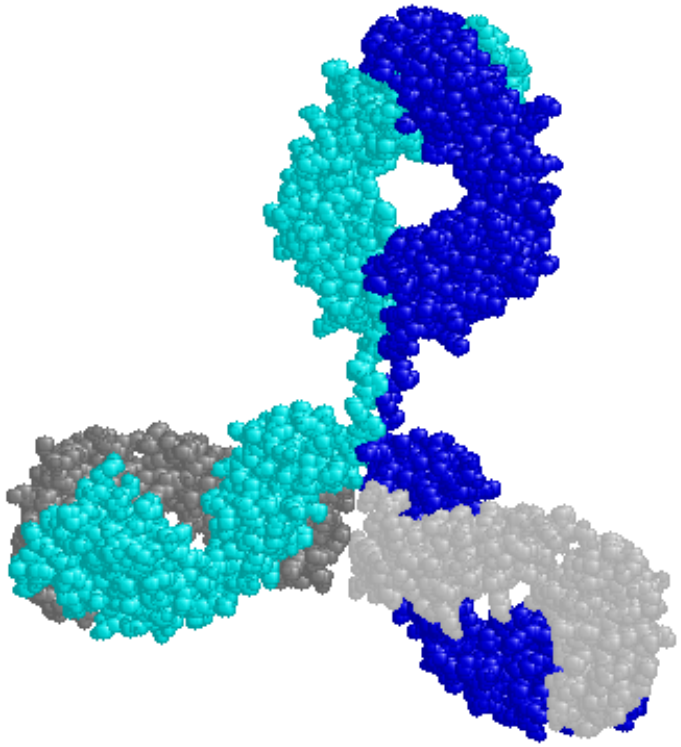
Signal Amplification

Signal Detection

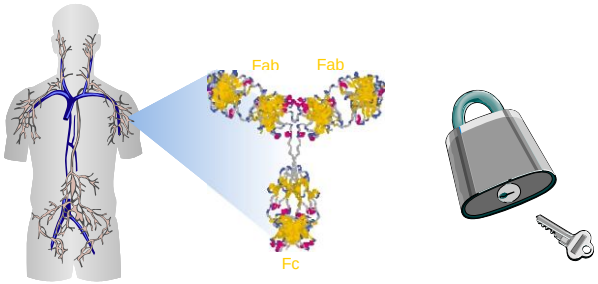
Part I:

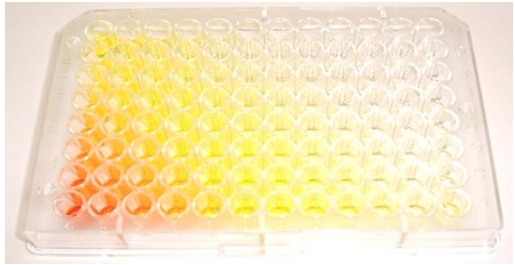
Protein-DNA Conjugates and Imperacer® Technology

Antibodies meets DNA:
Natures Key to Recognition & Amplification (I)



Antibodies meets DNA:
Natures Key to Recognition & Amplification (II)

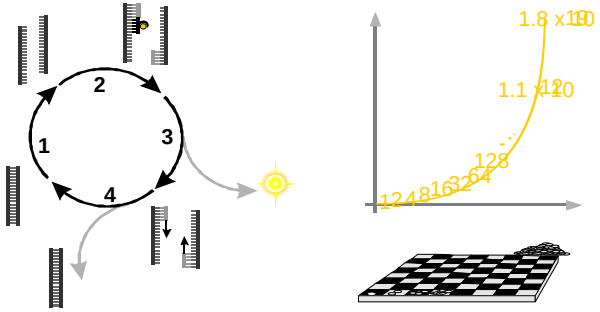


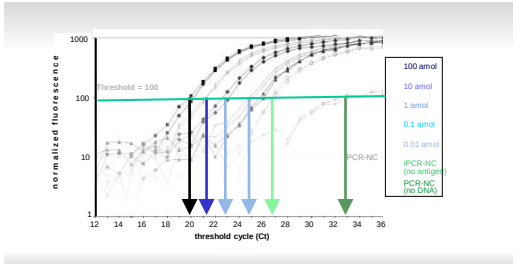


Antibody-antigen binding enables the immune system to recognize target compounds.

Immunoassays apply efficient antibodies for sample analysis

Sensitivity =
Specificity + Binding strength



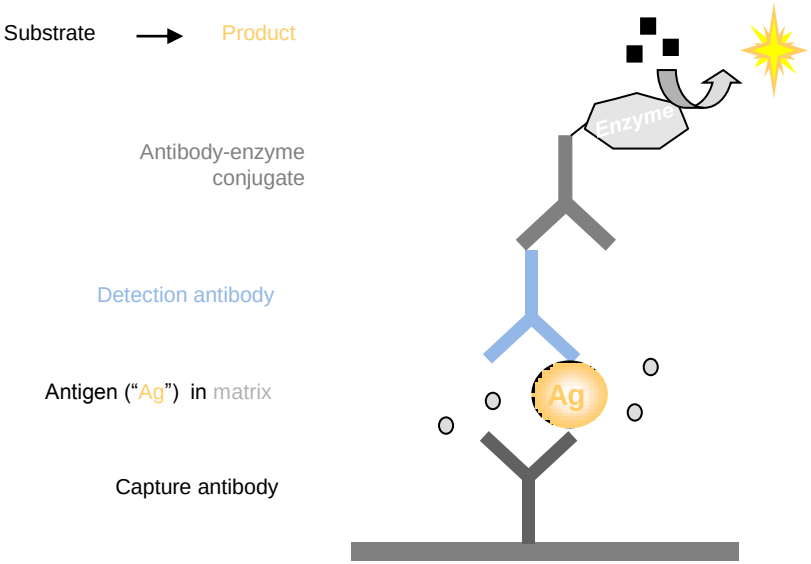


The duplication of stable DNA in cell replication is utilized for exponential target amplification.

Polymerase chain reaction (PCR) apply cyclic DNA amplification

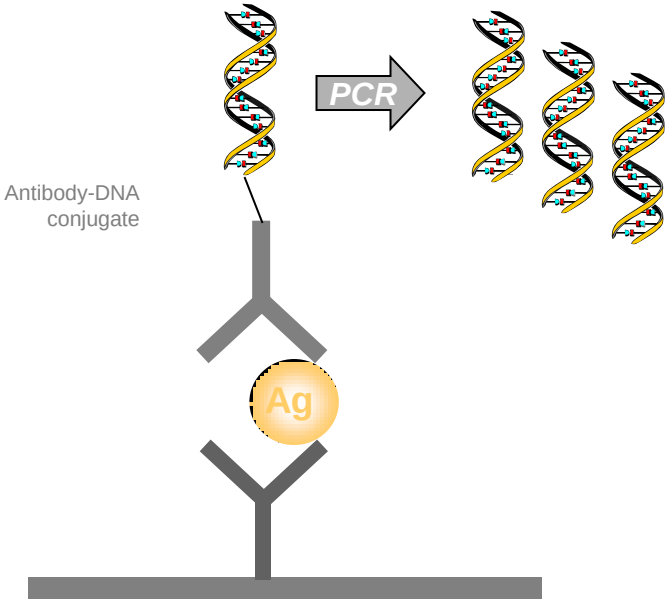
Sensitivity =
Efficient signal amplification

Sensitivity by Combination: Immuno-PCR



ELISA

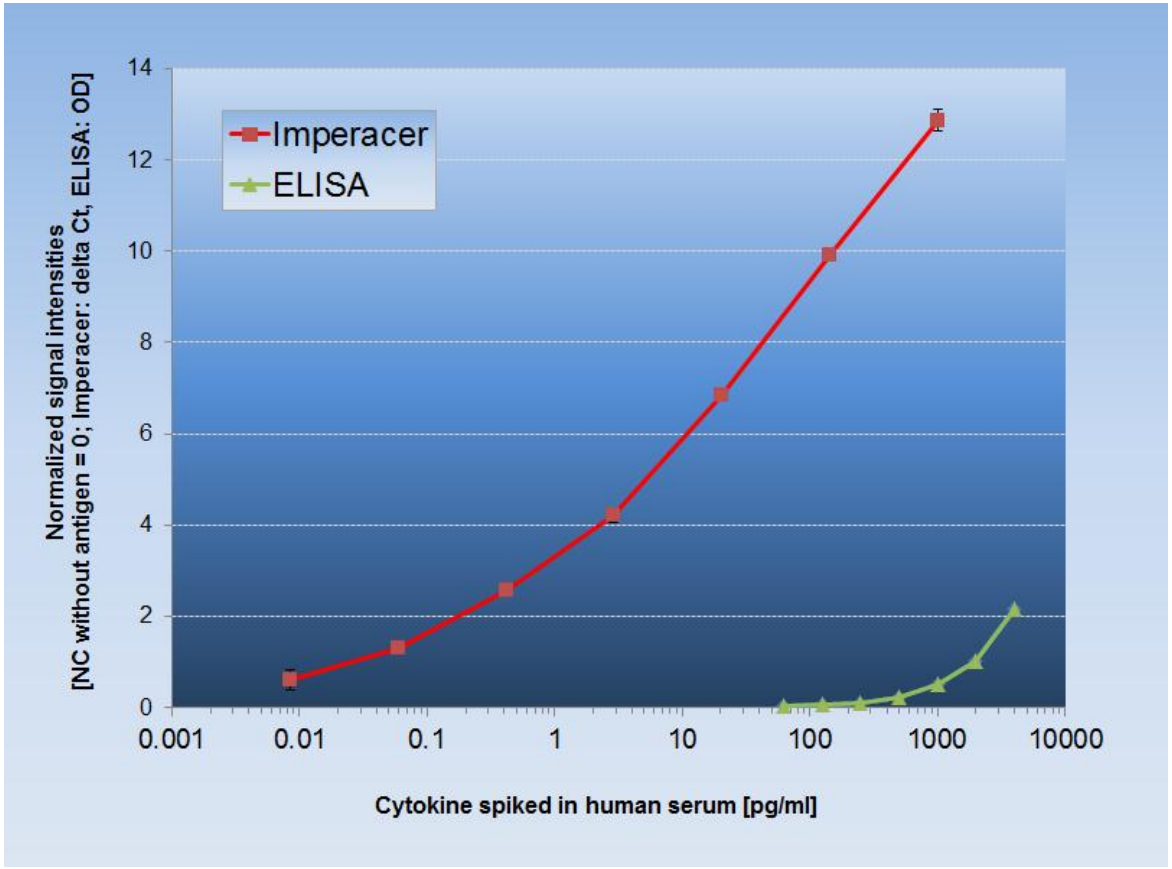
Detection by antibody-enzyme conjugates



Immuno-PCR

Detection by antibody-DNA conjugates

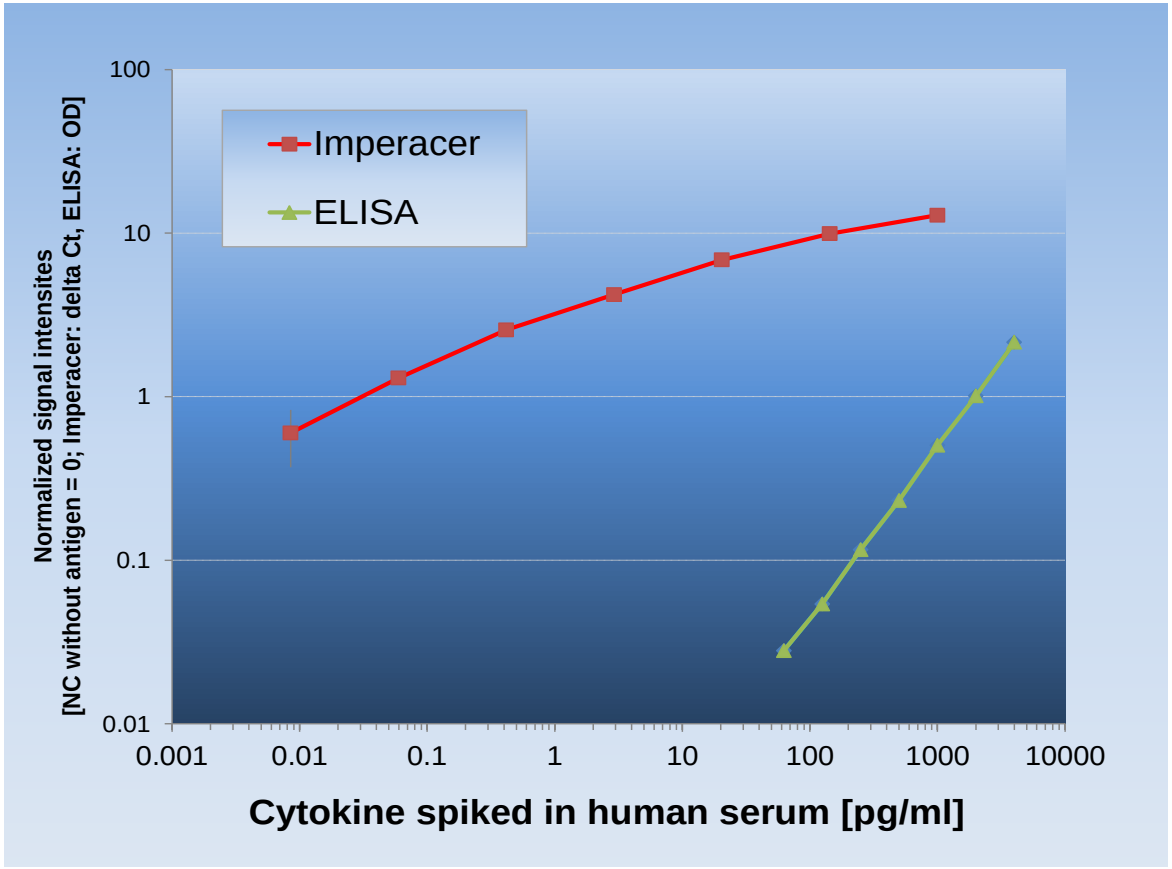
ELISA vs. IPCR



Exchange of the antibody-enzyme conjugate with an antibody-DNA conjugate

*Typically **1000 fold** increase in sensitivity*

ELISA vs. IPCR



Exchange of the antibody-enzyme conjugate with an antibody-DNA conjugate

*Typically **1000 fold** increase in sensitivity & increased dynamic range: Detect high and low cytokine levels in the same experiment.*

What is „*Imperacer®*“ ?

Tailored Reagents

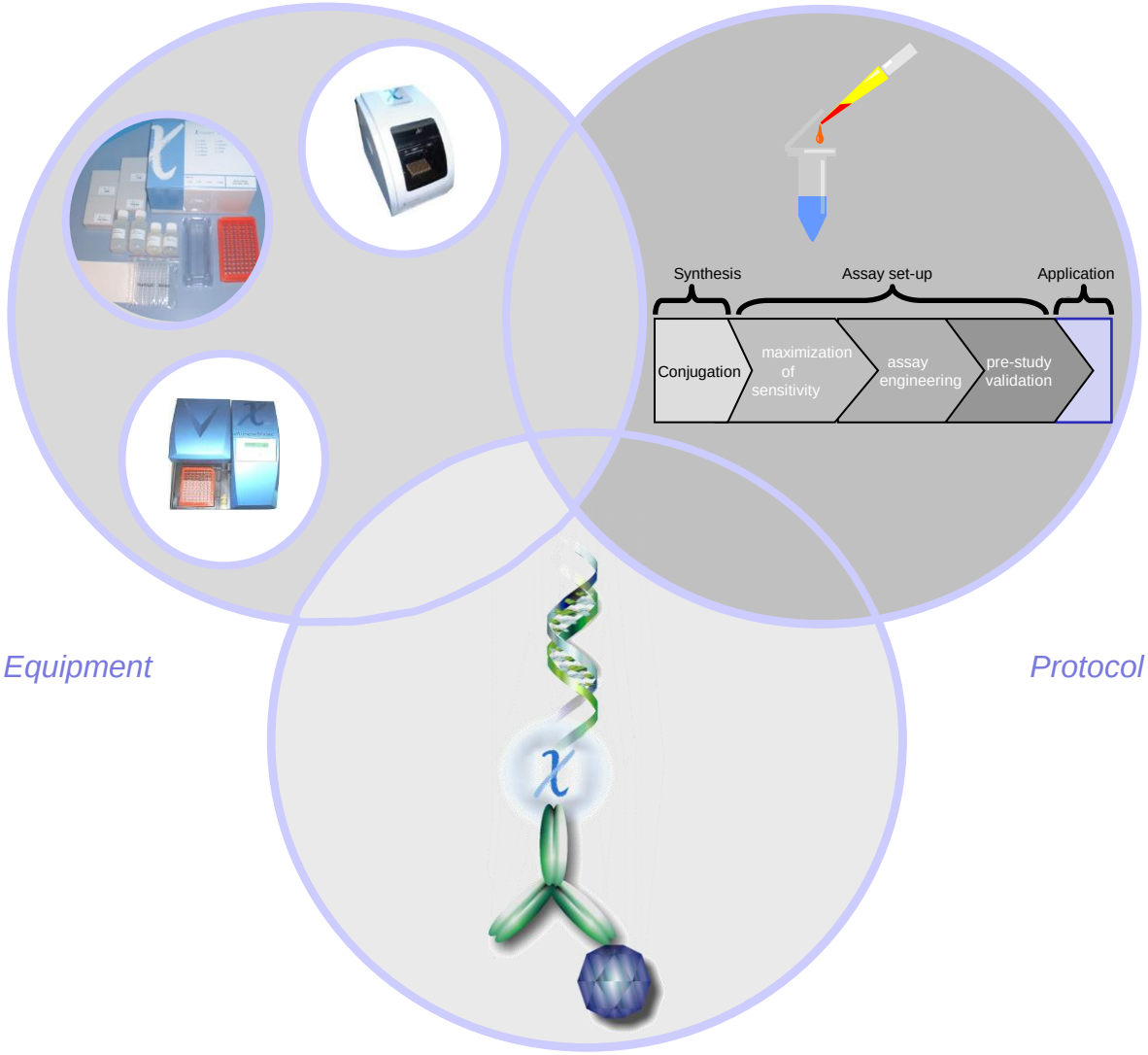
Optimized Protocol

Complete Work Station

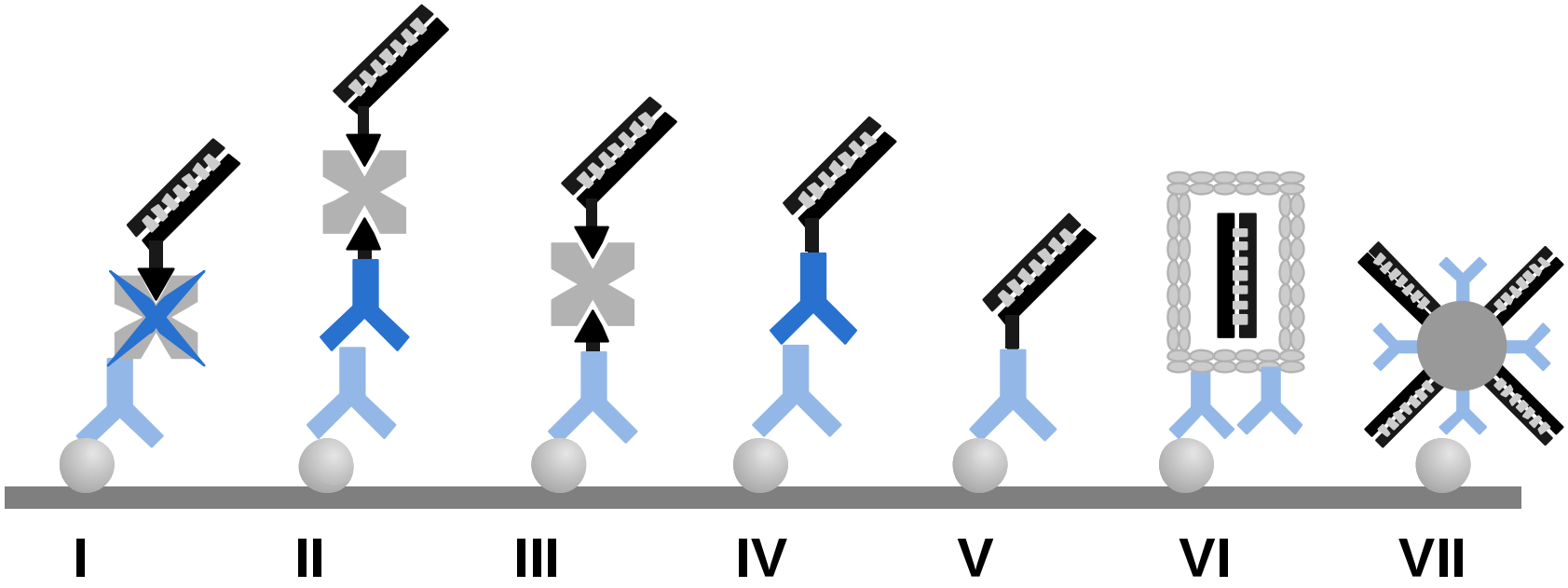
Assay Development Strategies& Expertise

Support Tools:

- AnySource®
- LIFE strategy (DOE)



Protein-DNA Conjugates as Key Elements for IPCR



I Sano et al.

STV-Protein A chimera

Science, 1992, 258(5079): 120

II Zhou et al.

Universal IPCR

NAR, 1993, 21(25): 6038

V Hendrickson et al.

Covalent conjugates

NAR, 1995, 23(3): 522.

Challenges of IPCR: *Link antibodies and DNA & detect DNA*

Evolution of the IPCR method from complex, multi-step sequential binding assays to ready-to-use antibody-DNA conjugates

What does „*Imperacer®*“ ?

High Sensitivity

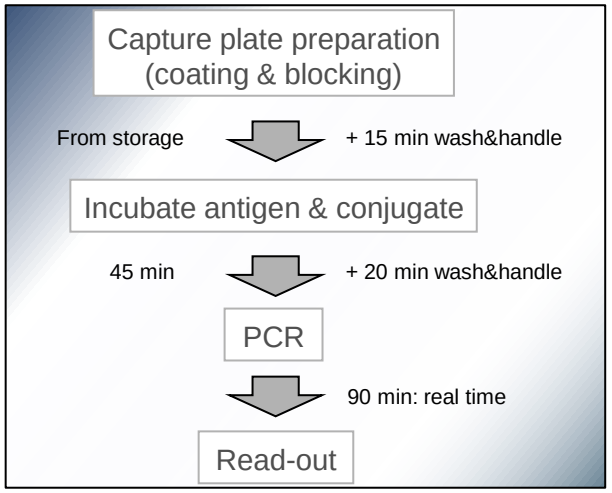
Matrix Robustness

Dynamic Range

Quantitative detection

ELISA Format

Optimized ready-to use Reagents & Easy Protocol



What does „*Imperacer®*“ ?

High Sensitivity

Matrix Robustness

Dynamic Range

Quantitative detection

ELISA Format

Optimized ready-to use Reagents & Easy Protocol

Sample dilution:

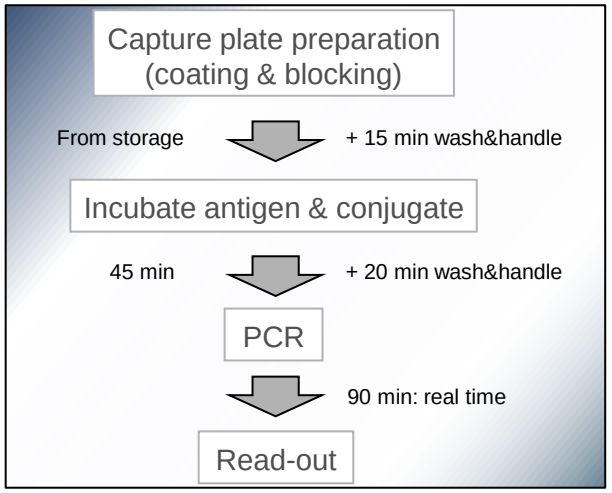
More & robust assays
from small volumes

Polyplex:

Muliple assays
from one sample

Antibody-Antigen-Coupling
and
Real-time PCR signal generation

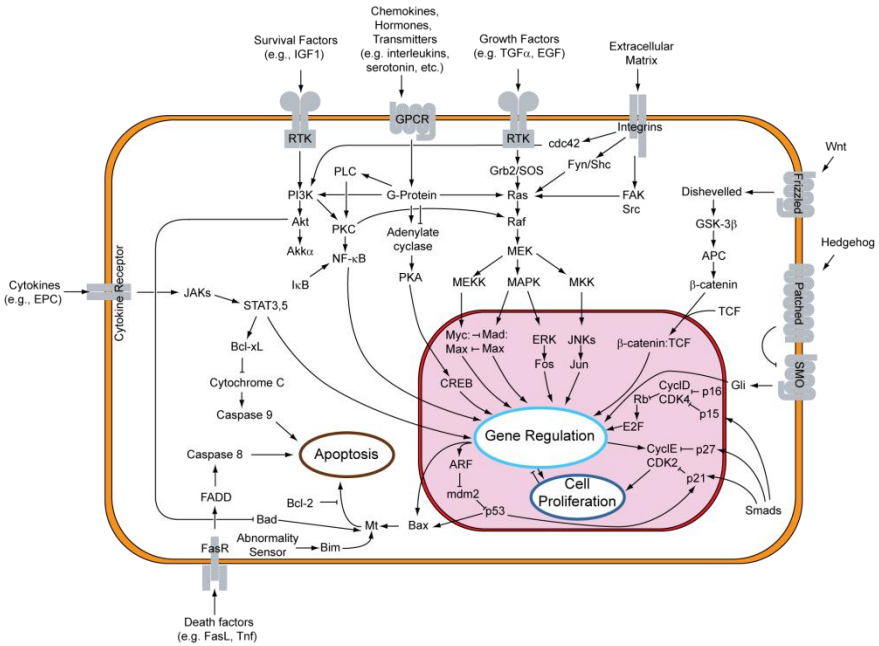
in one microplate format
(30 µl assay volume)

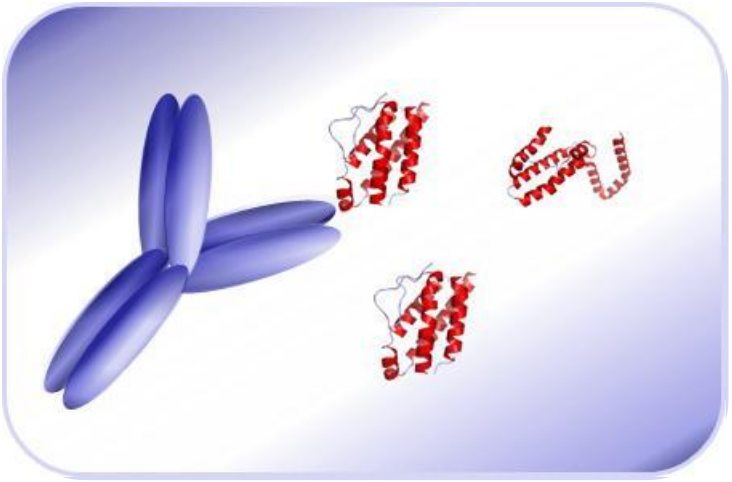


Part II:

The Challenges of Cytokine Detection

Interaction of Cytokines & Cytokine-binding Proteins

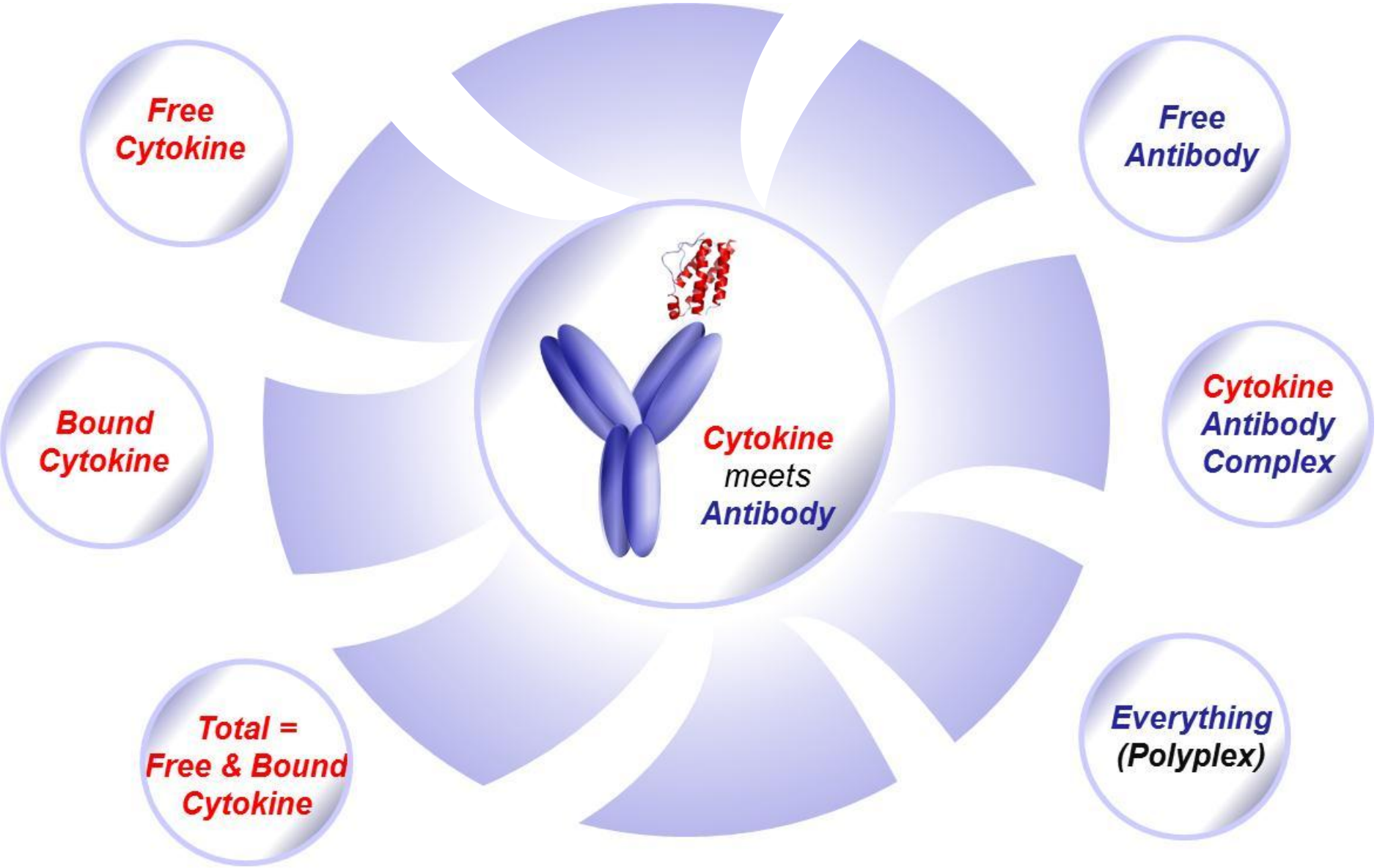




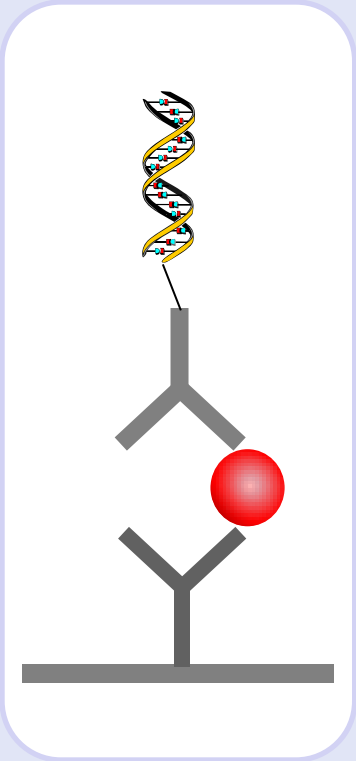
Cytokines have a key role in signal transmission, especially in immune response.

Regualtion of cytokine function by application of cytokine drugs and/or cytokine binding molecules (e.g. therapeuthic antibodies) is therefore a valuable tool for affecting inflammatory diseases.

Analytical Targets



Target 1: Free Cytokines

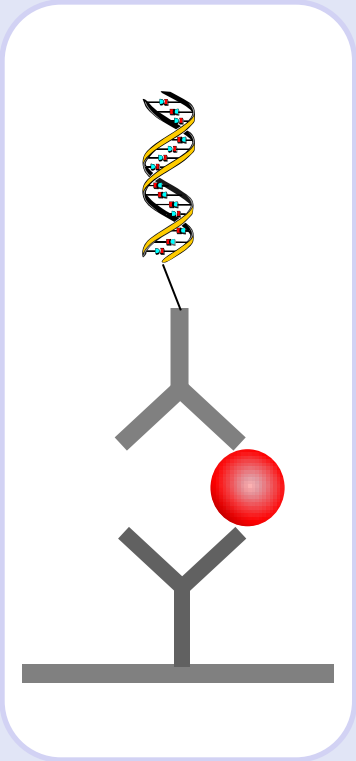


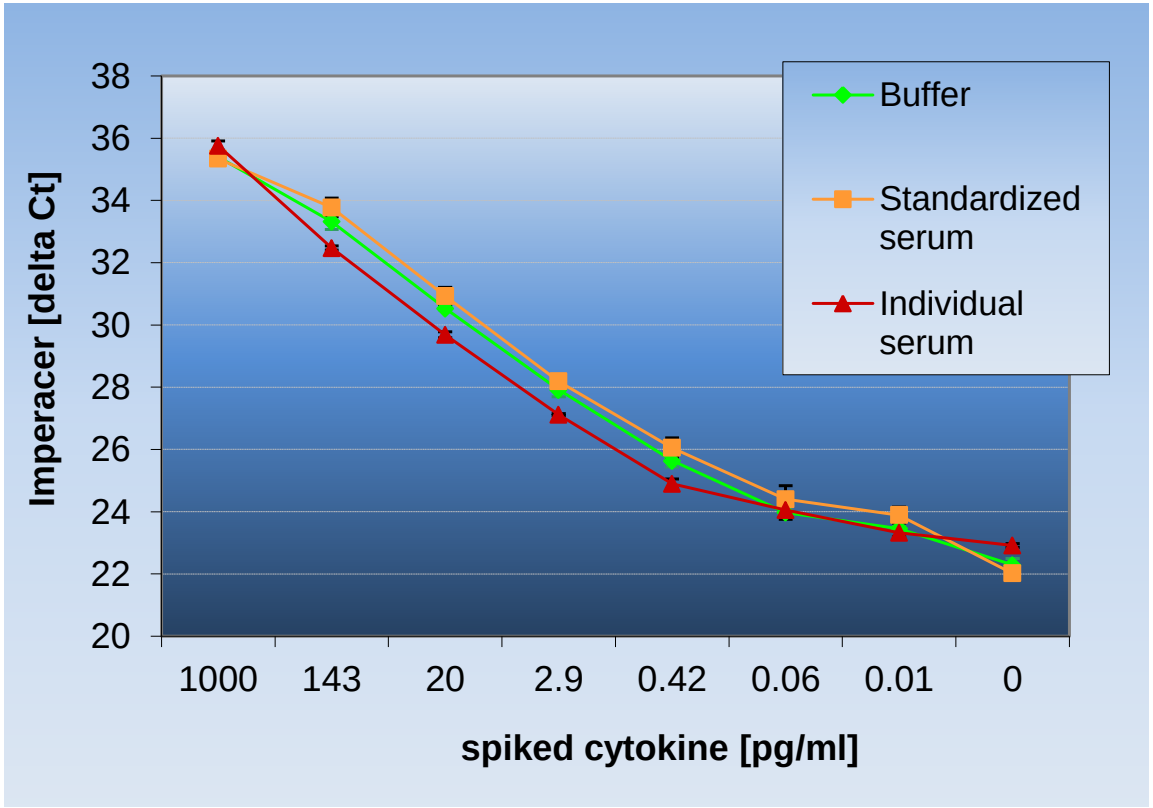
Imperacer® Limit of Detection for various Cytokines in spiked human serum

Il-2:	0.1 pg/ml	1.5 fg/well
IL-6:	0.1 pg/ml	1.5 fg/well
IL-8:	0.1 pg/ml	1.5 fg/well
IL-11:	0.8 pg/ml	12 fg/well
IL-12:	50 fg/ml	0.75 fg/well
IL-17:	20 fg/ml	0.5 fg/well
IL-20:	50 fg/ml	0.75 fg/well
IL-21:	0.2 pg/ml	3 fg/well
IL-23:	0.2 pg/ml	3 fg/well
INFγ:	0.01 pg/ml	0.15 fg/well
TNFα:	10 fg/ml	0.3 fg/well
TNFα $_{\text{in buffer}}$:	1 fg/ml	0.03 fg/well

Note: Not all assays were fully optimized & not all assays apply similar sample dilution. Concentrations are provided for the spiked, non-diluted serum; absolute amounts/well include sample dlution.

Target 1: Free Cytokines



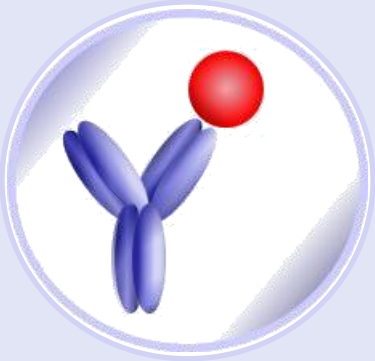


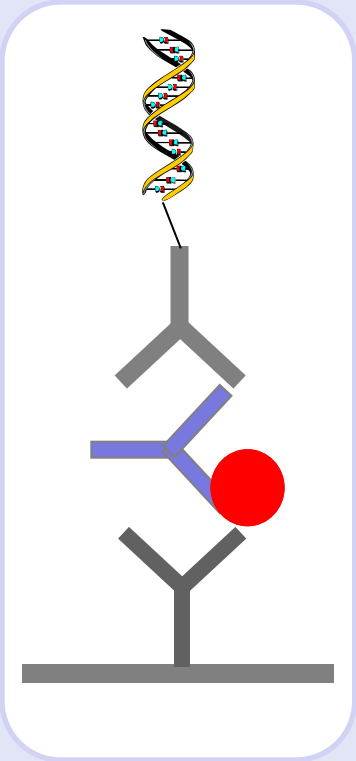
Broad dynamic range of Imperacer®: >4 orders of magnitude

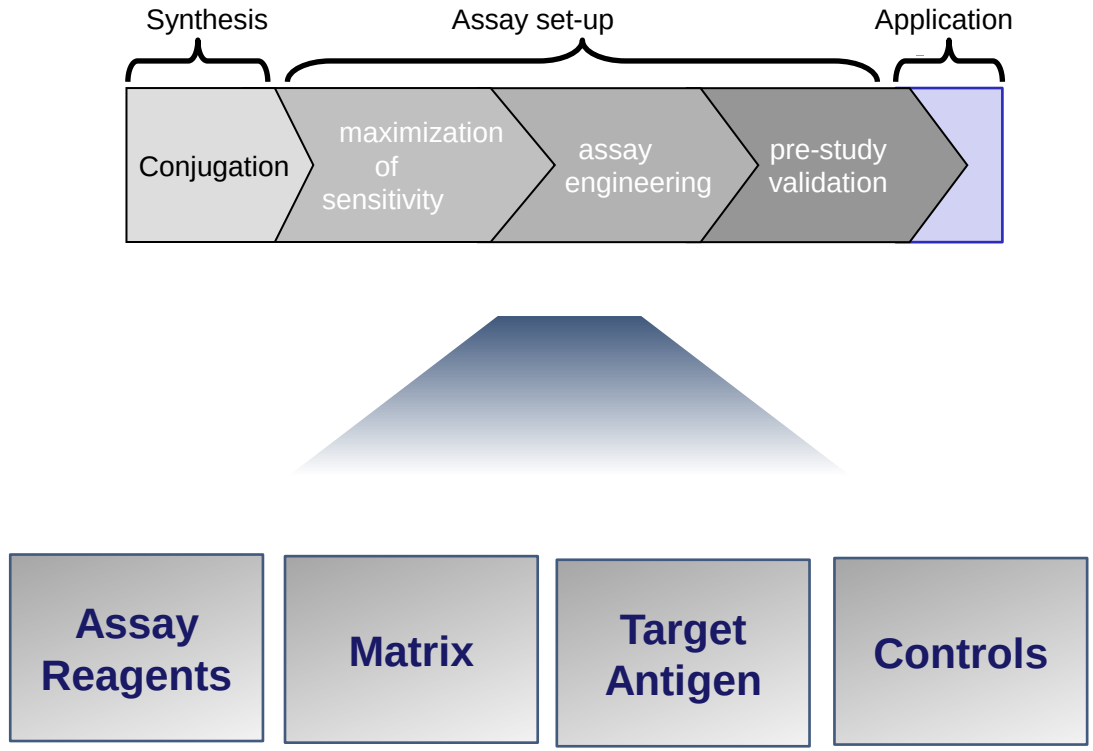
Assay development with increasing matrix complexity:

High robustness in different matrices - Standardized matrices for reference controls
(sample diluted 1+1 in tailored AnySource® buffer)

Target 2: Cytokine-Antibody Complex



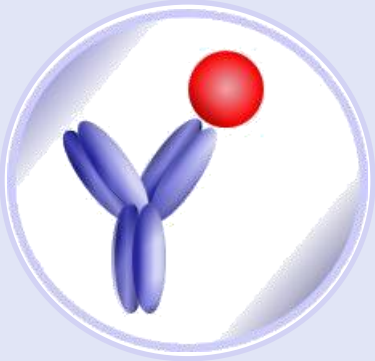


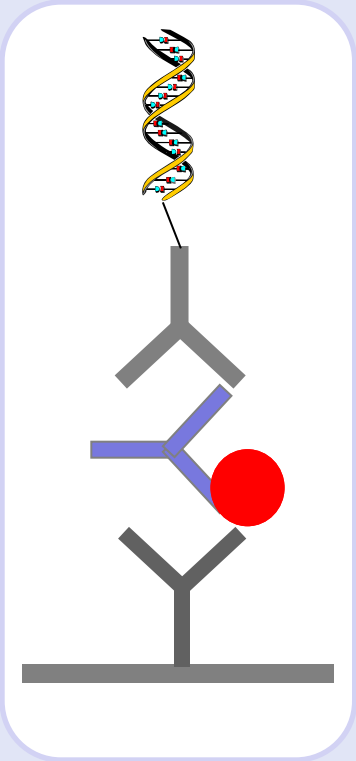


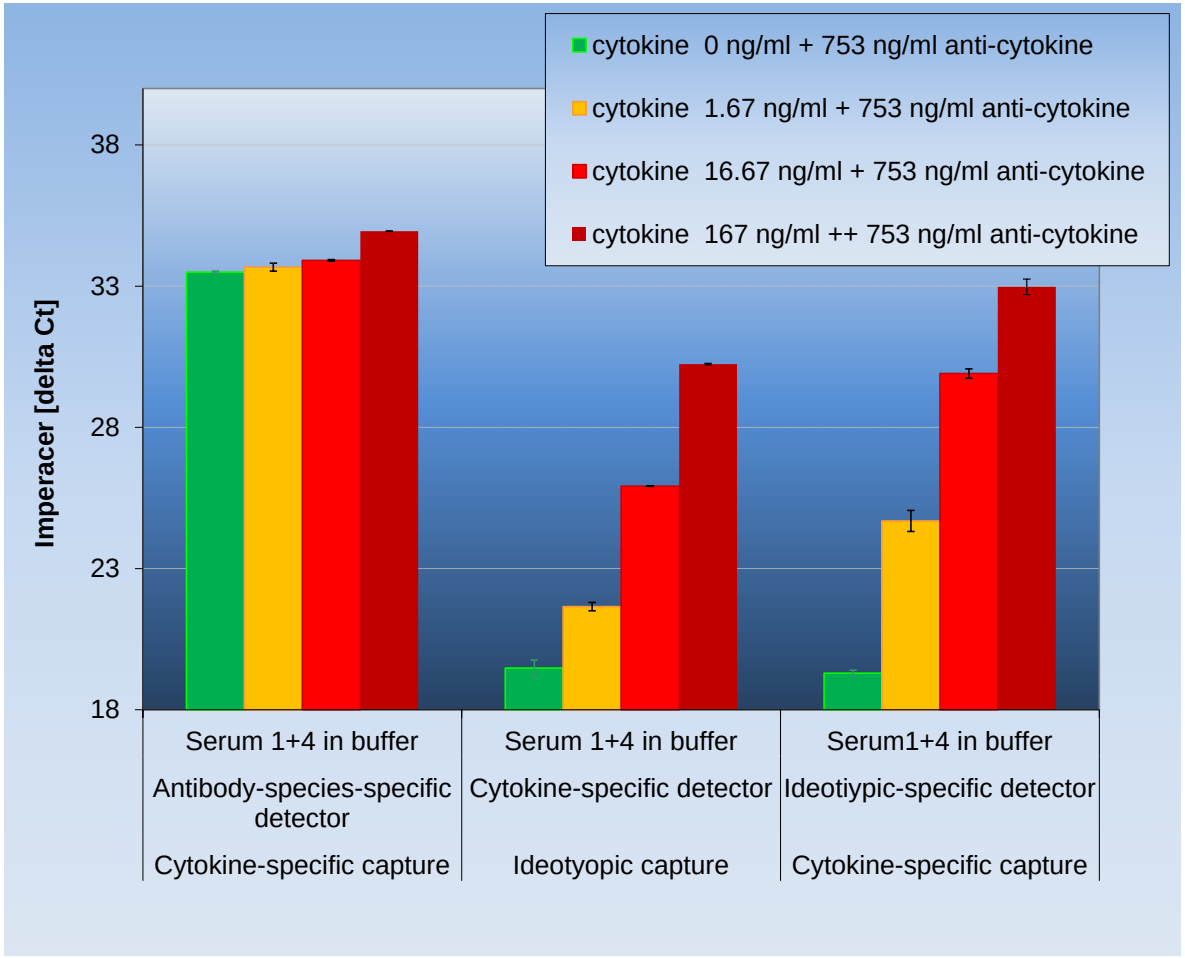
Assay development and optimization

Different assay challenges are addressed during adaptation of the protocol to the intended target.

Target 2: Cytokine-Antibody Complex



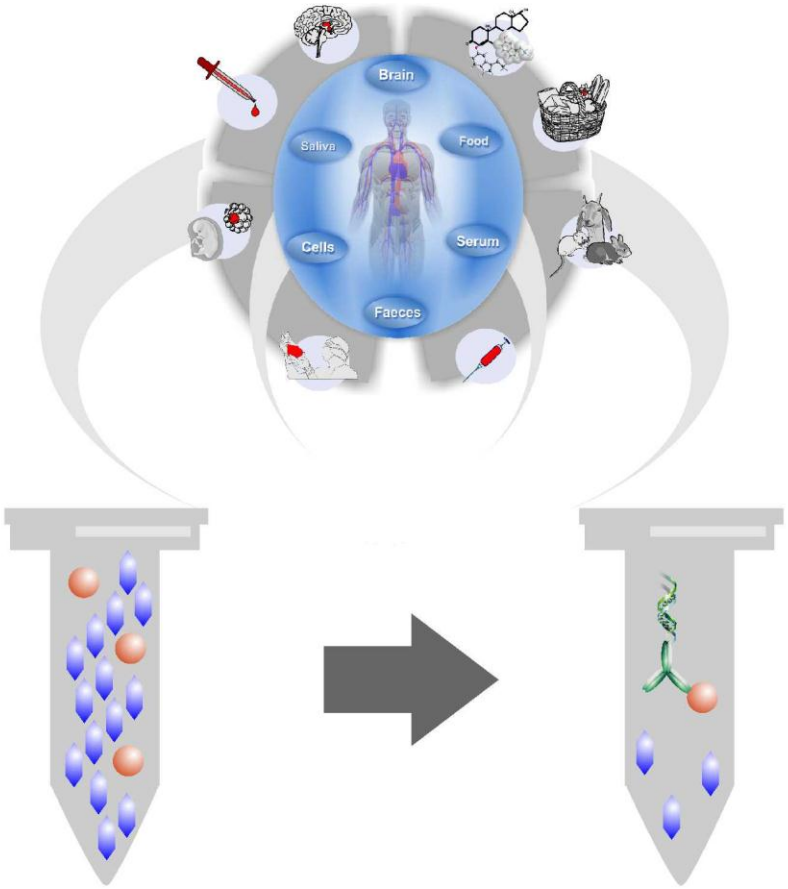




Challenge 1: Reagent Selection

Test of different combinations of cytokine-specific or idiotypic capture antibodies and cytokine-specific, species-specific or idiotypic detection conjugates.
Best results were obtained with idiotypic antibodies.

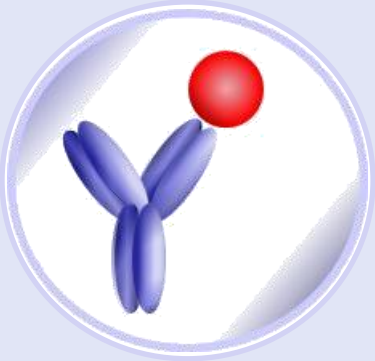
AnySource® Sample Dilution for Assay Robustness

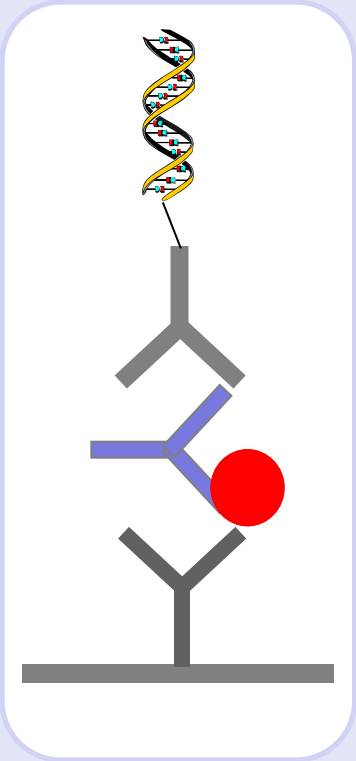


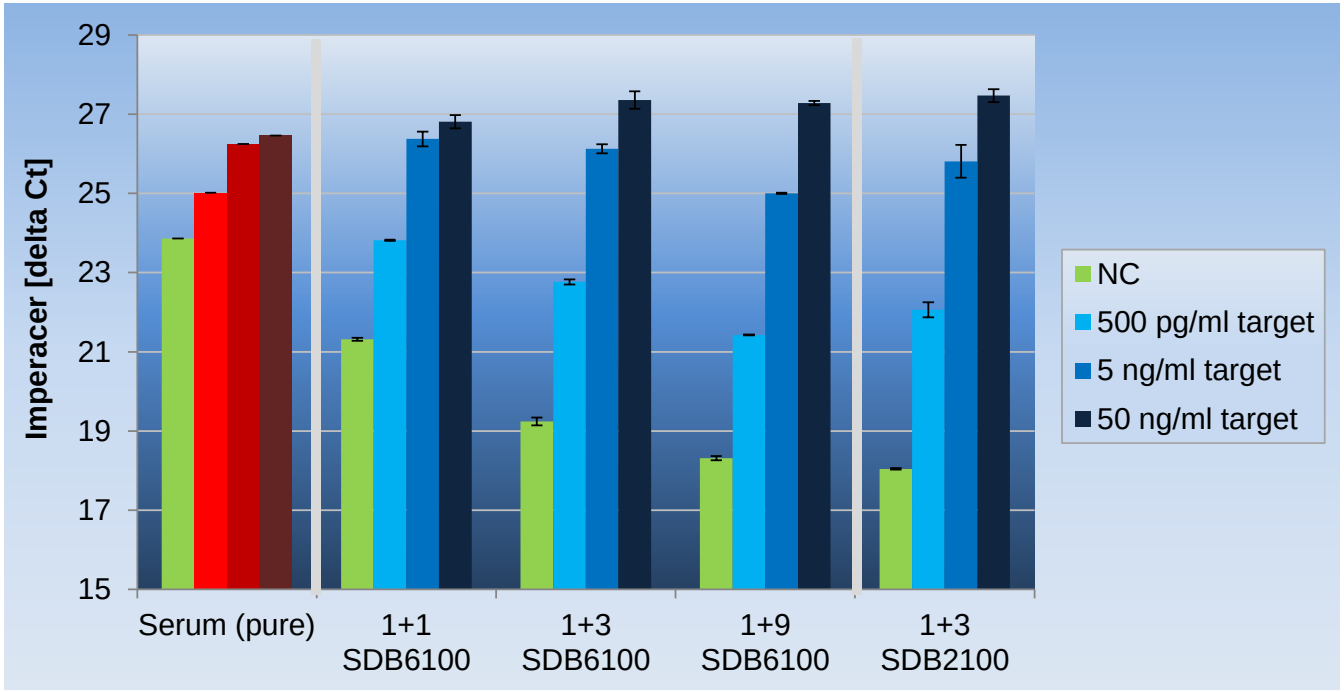
Biological Sample:
Matrix compounds ( )
interfere with target ( )
detection

Sensitive IPCR
target detection after
sample dillution

Target 2: Cytokine-Antibody Complex



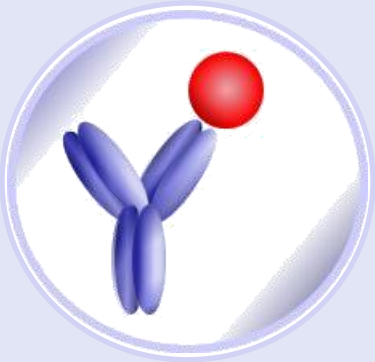


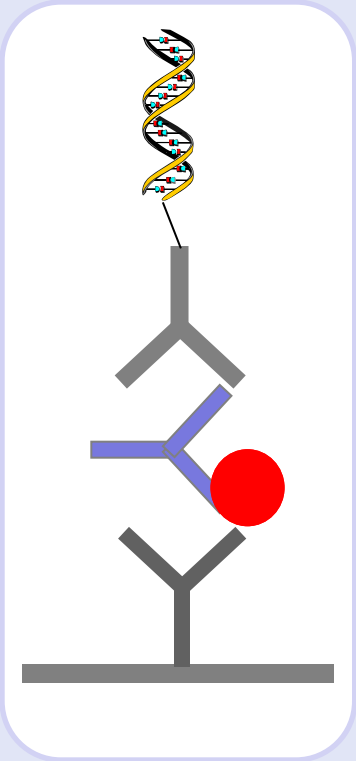


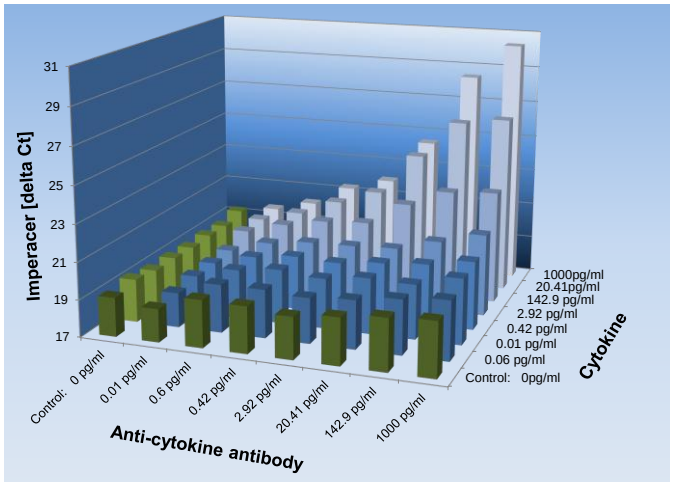
Challenge 2: Matrix Adaptation

If interfering compounds in biological matrix are present (e.g. native IgG in serum and no available idiotiypic detection antibody against the anti-cytokine antibody), tailored sample dilution in AnySource® sample dilution buffers („SDB“) compensates unspecific interaction.

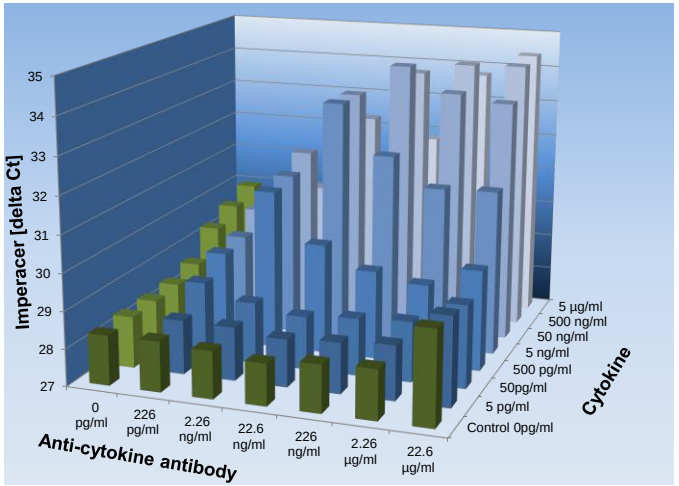
Target 2: Cytokine-Antibody Complex







Cytokine 1 +
Anti-cytokine 1



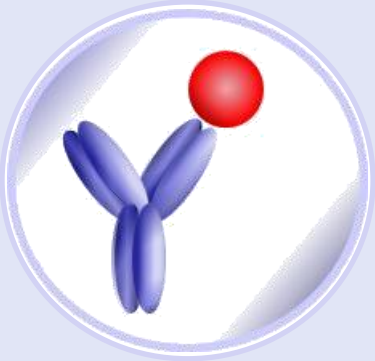
Cytokine 2 +
Anti-cytokine 2

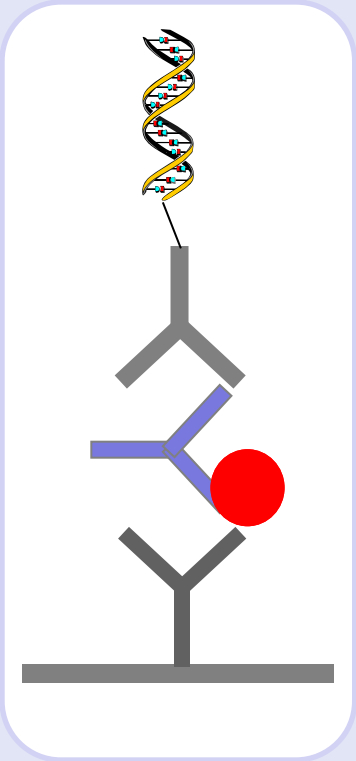
Challenge 3: Complex Formation

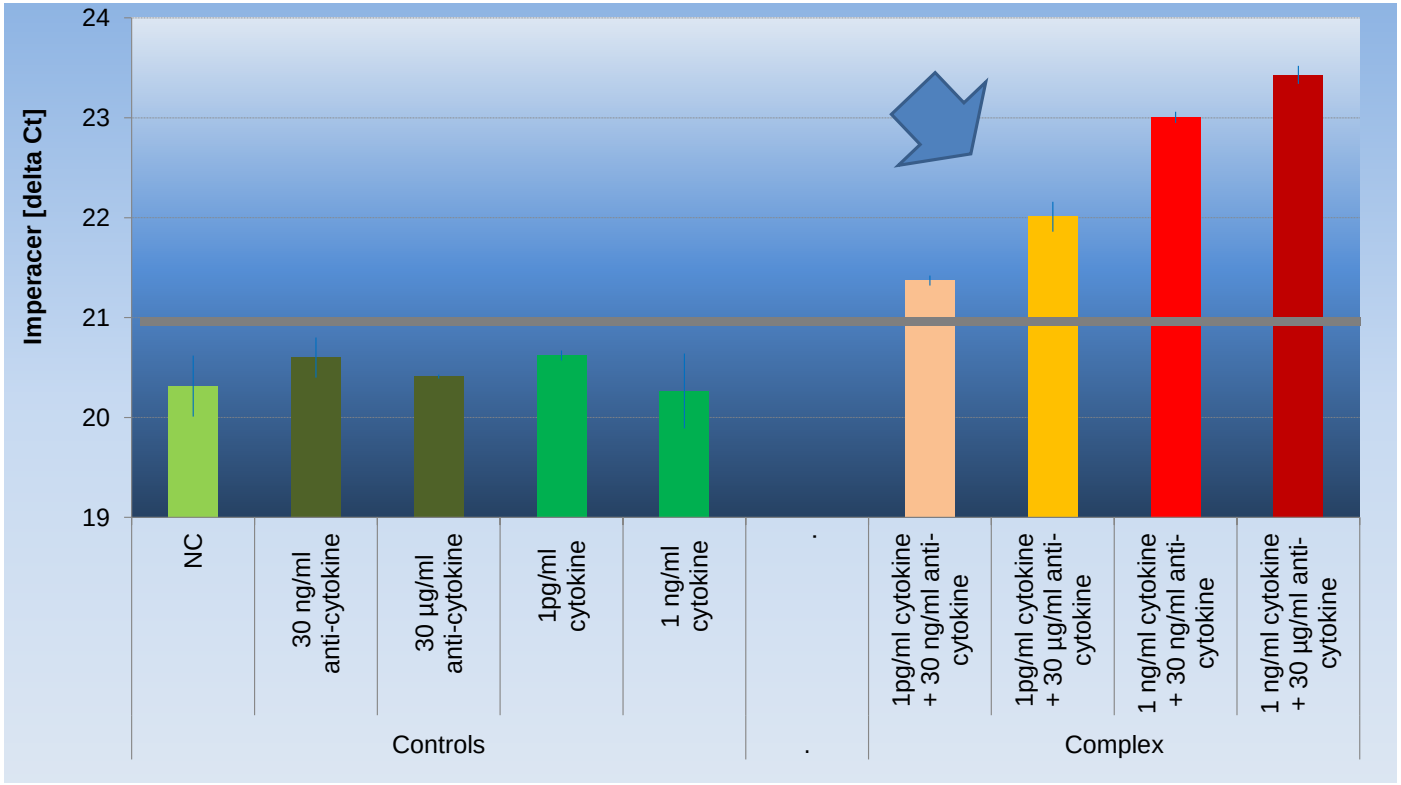
Different mixture ratios of cytokine and anti-cytokine antibody in a systematic concentration grid revealed different complex compositions & a specific signal intensity pattern for the complex population.

Only massive overload of one compound induces unspecific response.
The signal therefore indicates the specific presence of the target complex and the qualitative amount of complex. However, exact composition of the target complex cannot be accessed by signal intensity alone.

Target 2: Cytokine-Antibody Complex



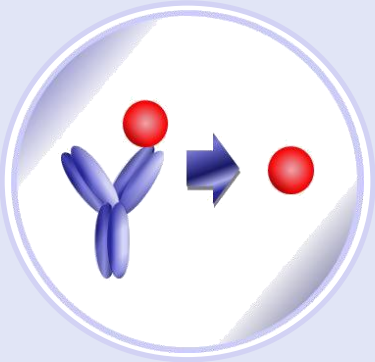


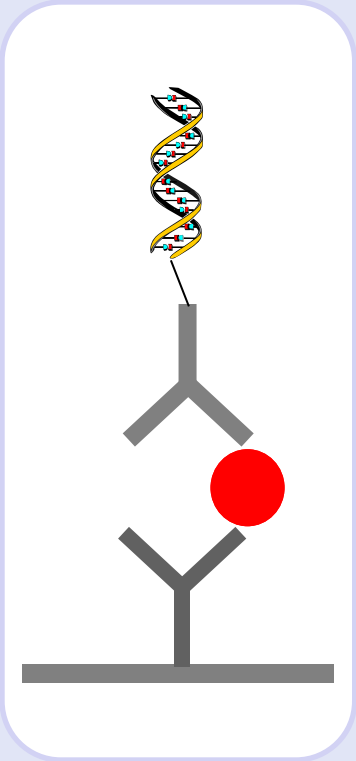


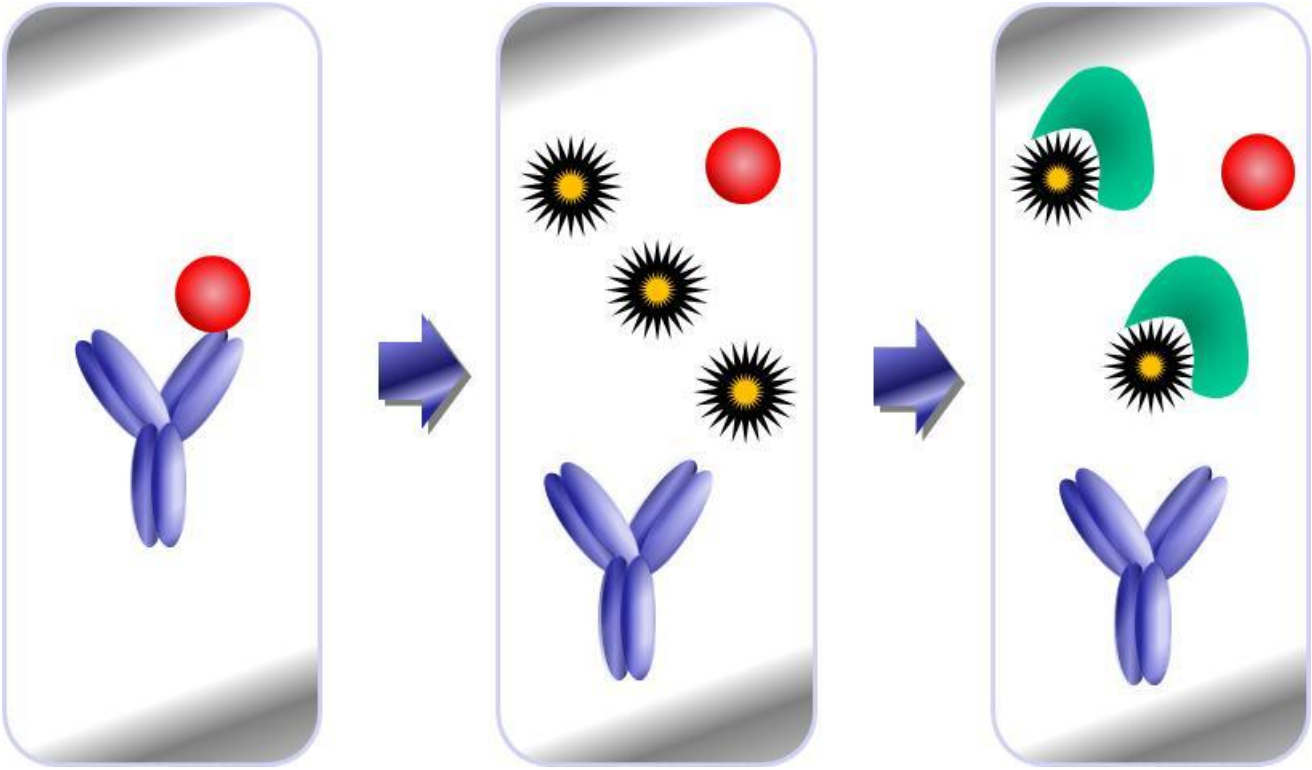
Challenge 4: Optimized Assay & Valid Data

Assay performance is tested against positive and negative controls in serum. A sensitive and specific detection of complex formation was demonstrated in the presence of excess cytokine or anti-cytokine, simulating the conditions in the native target enviroment.

Target 3: Bound Cytokines



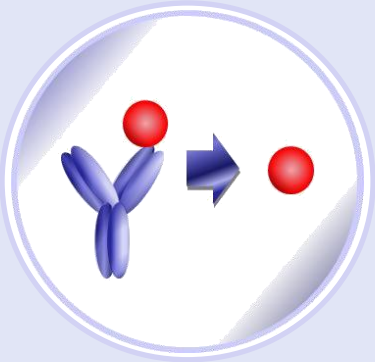


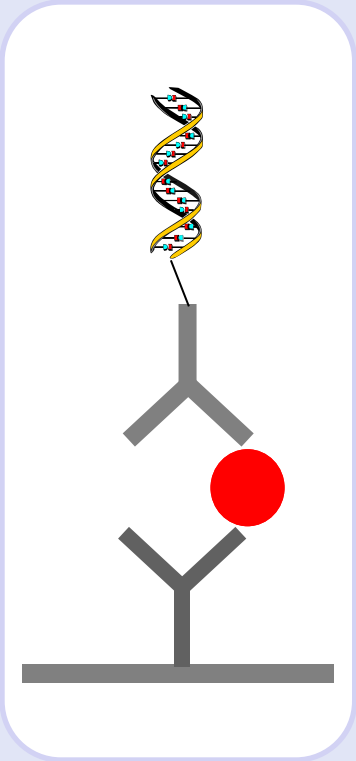


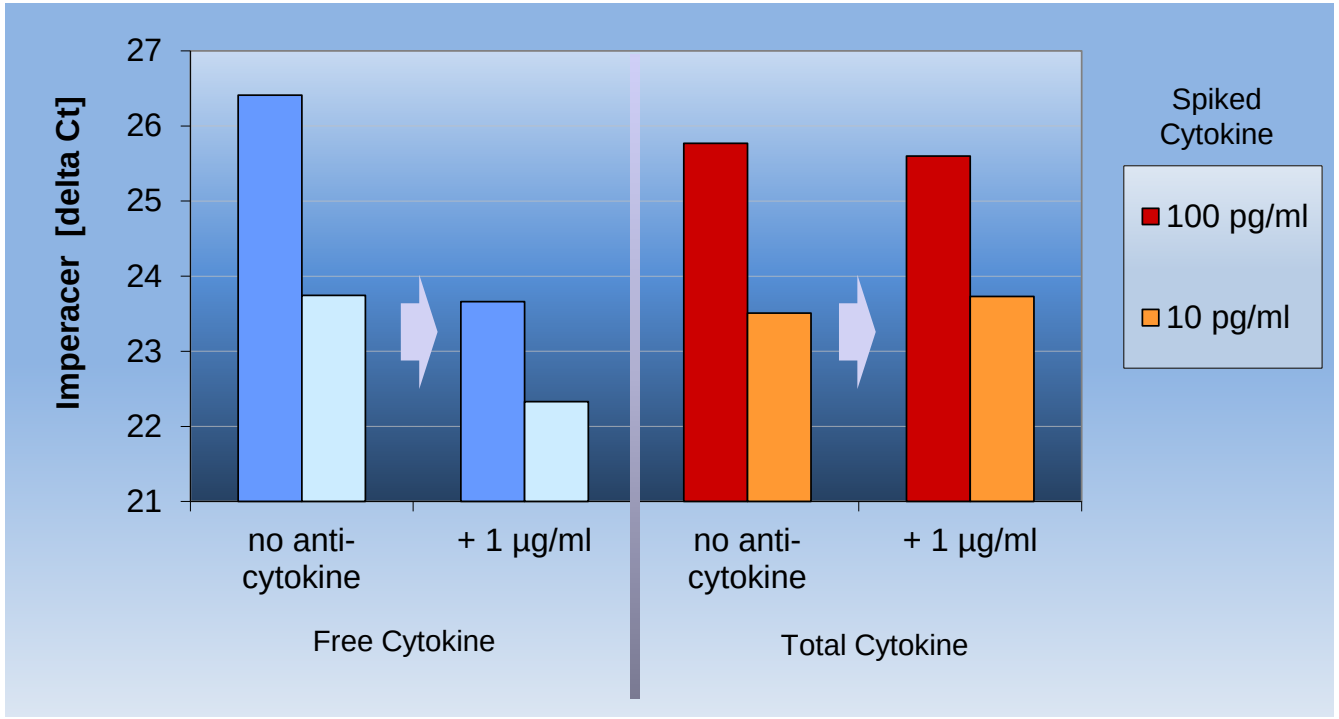
Enforced Dissociation: Separation of Bound Cytokine & anti-Cytokine

By addition of a dissociating agent (organic solvent, acid, detergent), bound cytokine is released from its binding partner. No additional purification is required as the dissociation reagent is neutralized by sample dilution.

Target 4: Total Cytokines





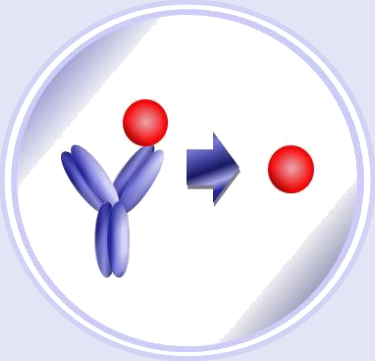


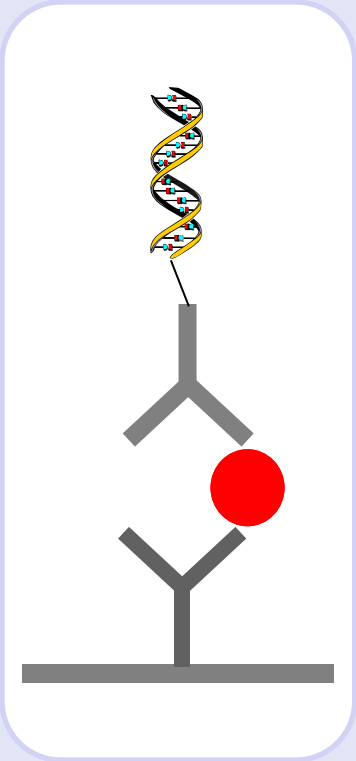
Free cytokine detection (blue) is influenced by the presence of anti-cytokine antibody.

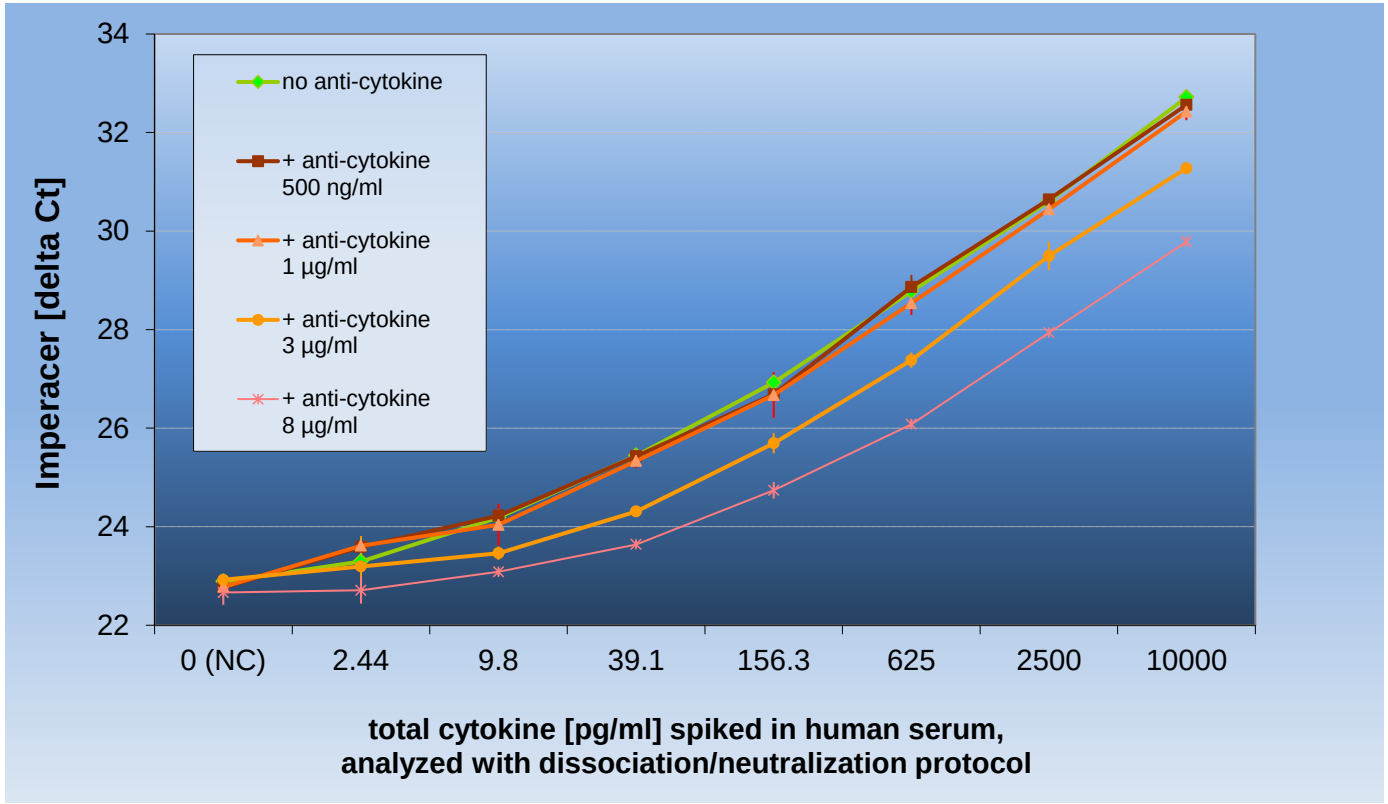
Total cytokine detection (red) compensates the interfering effect by dissociation / neutralization and gives constant values for cytokine concentrations in the absence&presence of interfering anti-cytokine antibody.

The enforced dissociation therfore enables the stable detection of
Free + **Bound** = **Total** Cytokine Concentration

Target 4: Total Cytokines



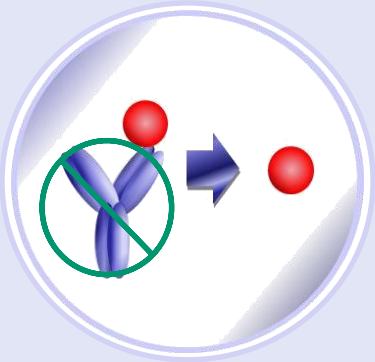


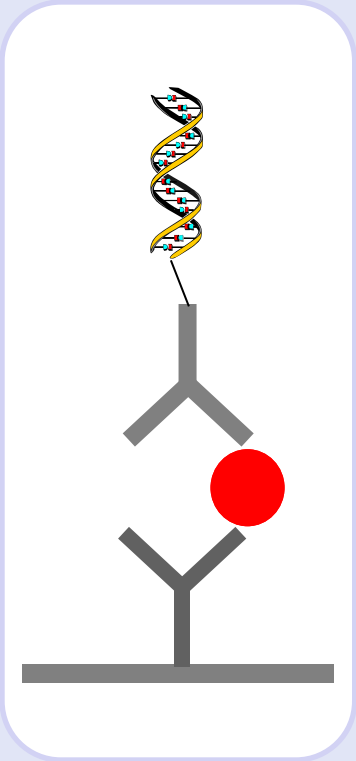


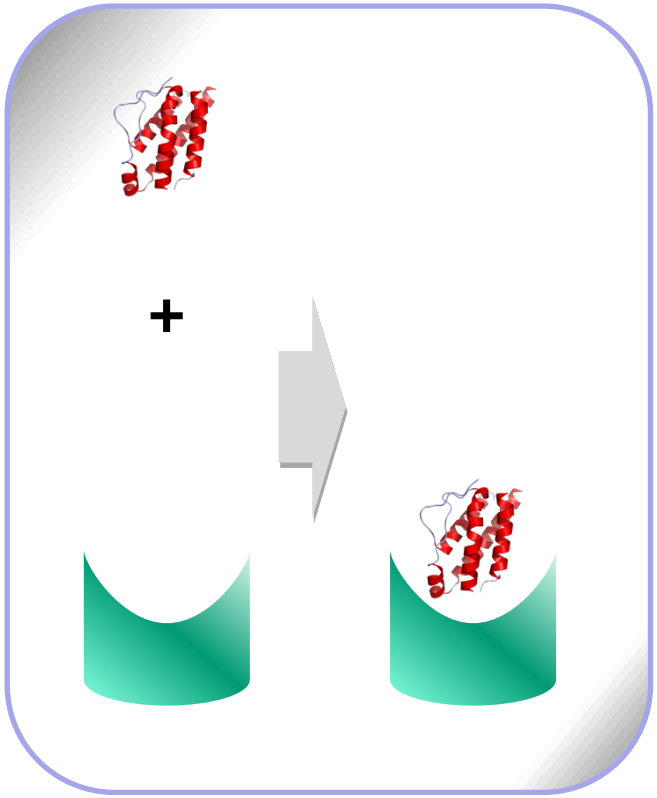
Stable and robust detection of cytokines in the presence of >100,000fold excess of anti-cytokine binding reagents:

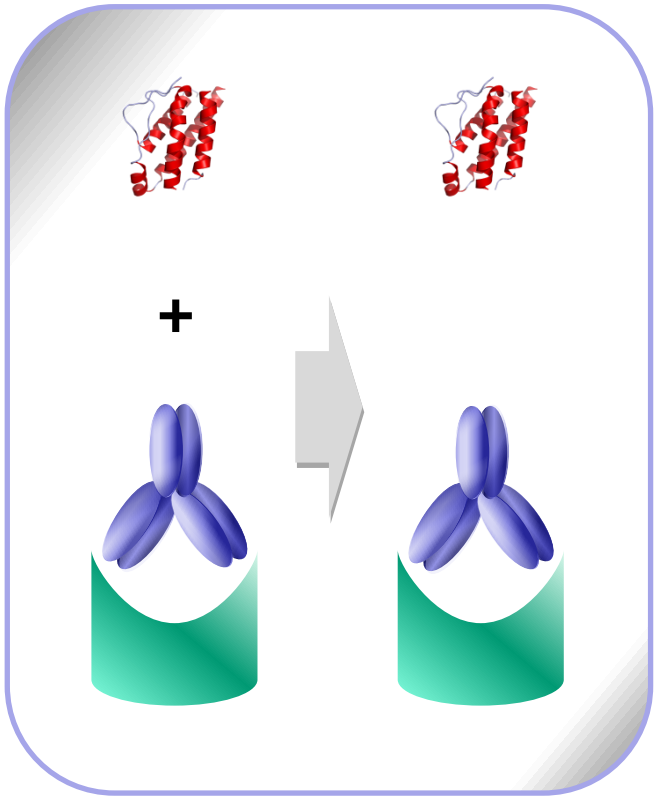
Cytokine detection was not influenced by the presence of up to 1 µg/ml interfering antibody and is still accessible with a broad dynamic range in the presence of 8 µg/ml interfering antibody.

Target 5: Cytokine Level Alteration by Receptor Blocking





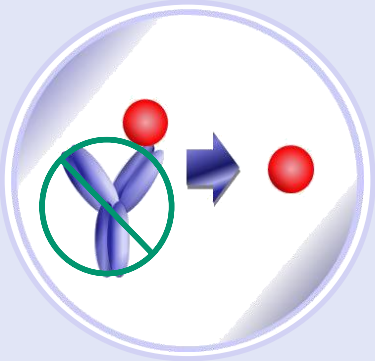


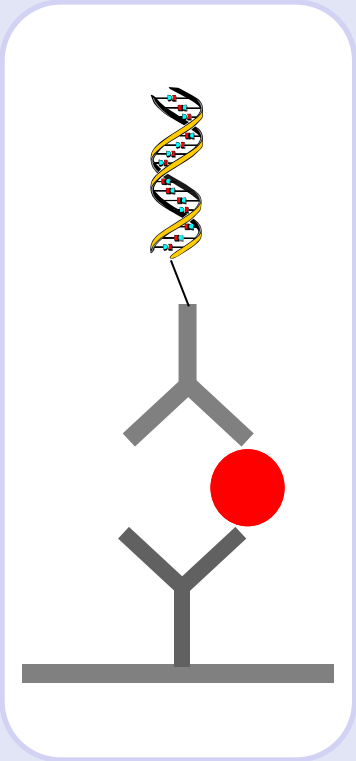


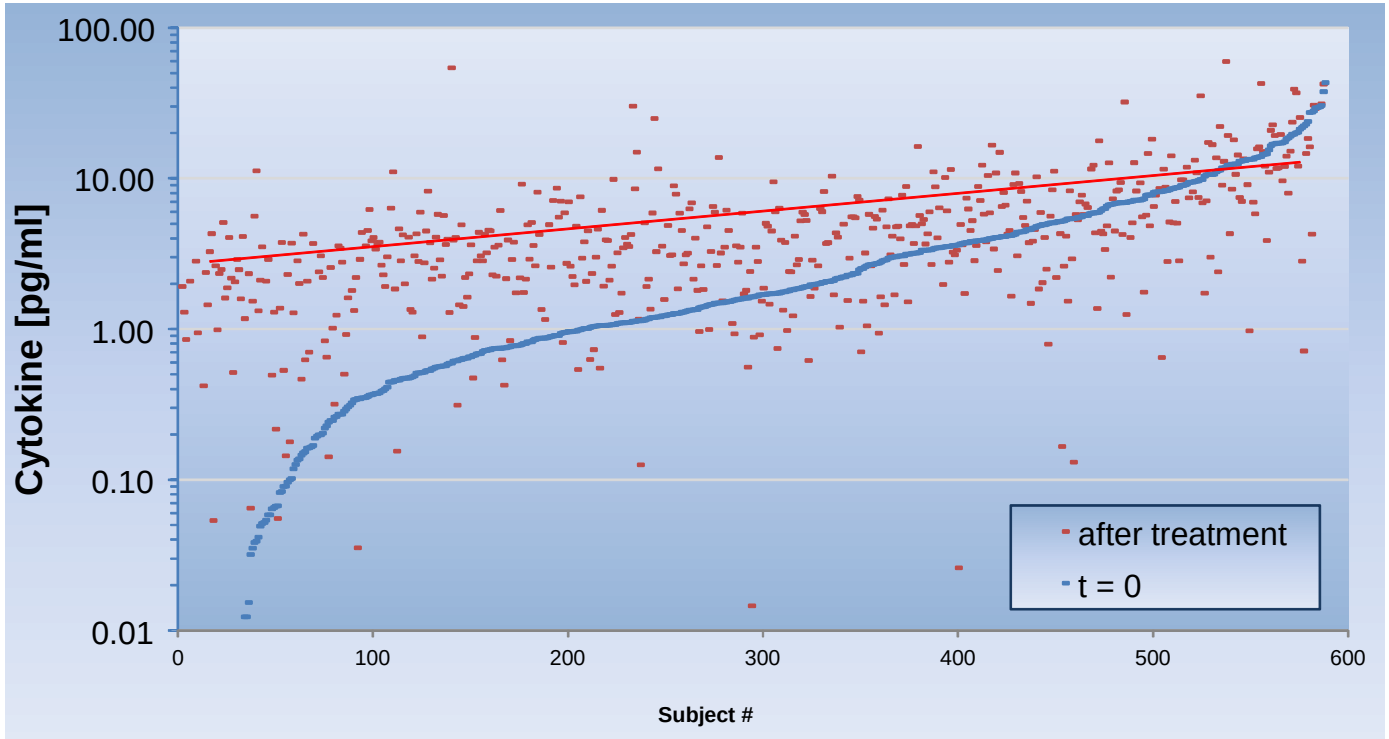
Native *cytokine binding receptors* can be blocked by *receptor-specifc drugs* (e.g. therapeuthic antibodies.

The interfering with cytokine-binding receptors influences the level of *bound and free cytokine* in native matrices.

Target 5: Cytokine Level Alteration by Receptor Blocking





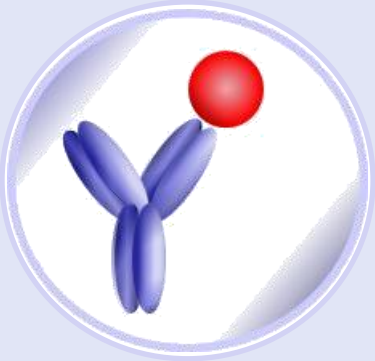


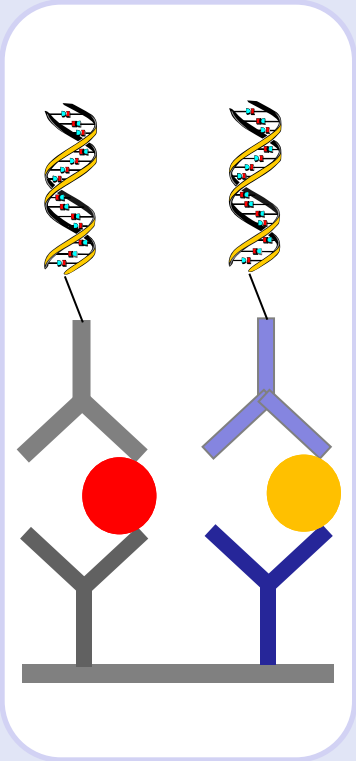
Clinical study: Application of a Cytokin-Binding-Receptor Inhibiting Drug

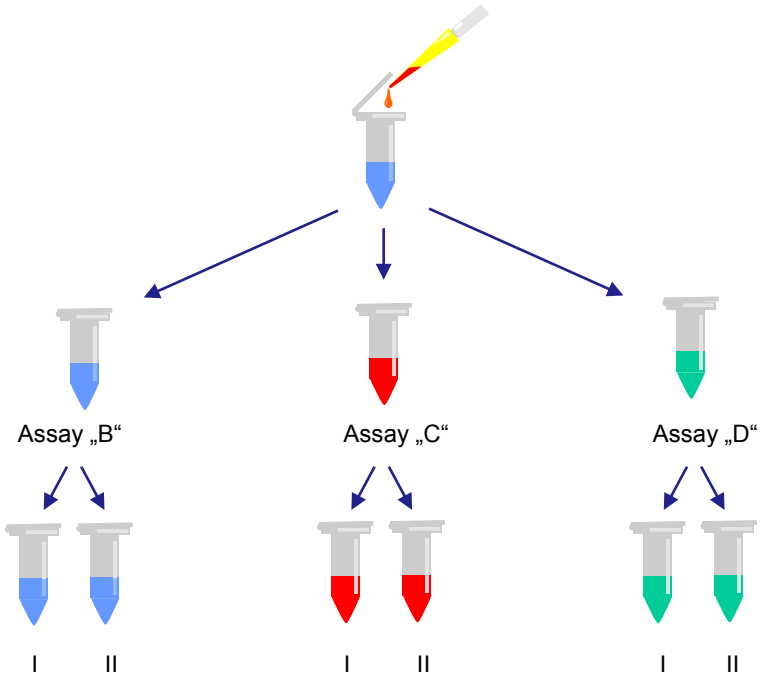
Imperacer® monitored the native cytokine level between <0.1 and approx. 50 pg/ml before treatment (blue) an the elevated cytokine level after treatment (red) in a panel of 500+ individuals.

Analysis of individual cytokine levels in serum after treatment with the drug revelaed an increased level of free cytokine which was no longer able to bind to the blocked receptor.

Target 6: Polyplex



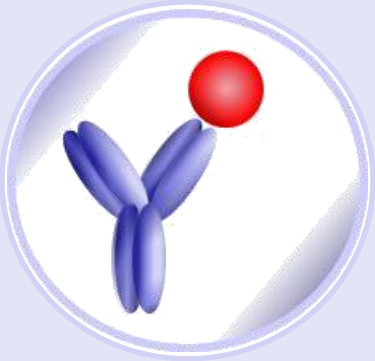


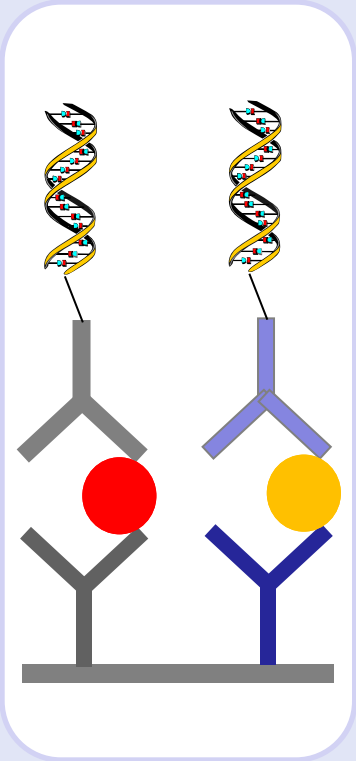


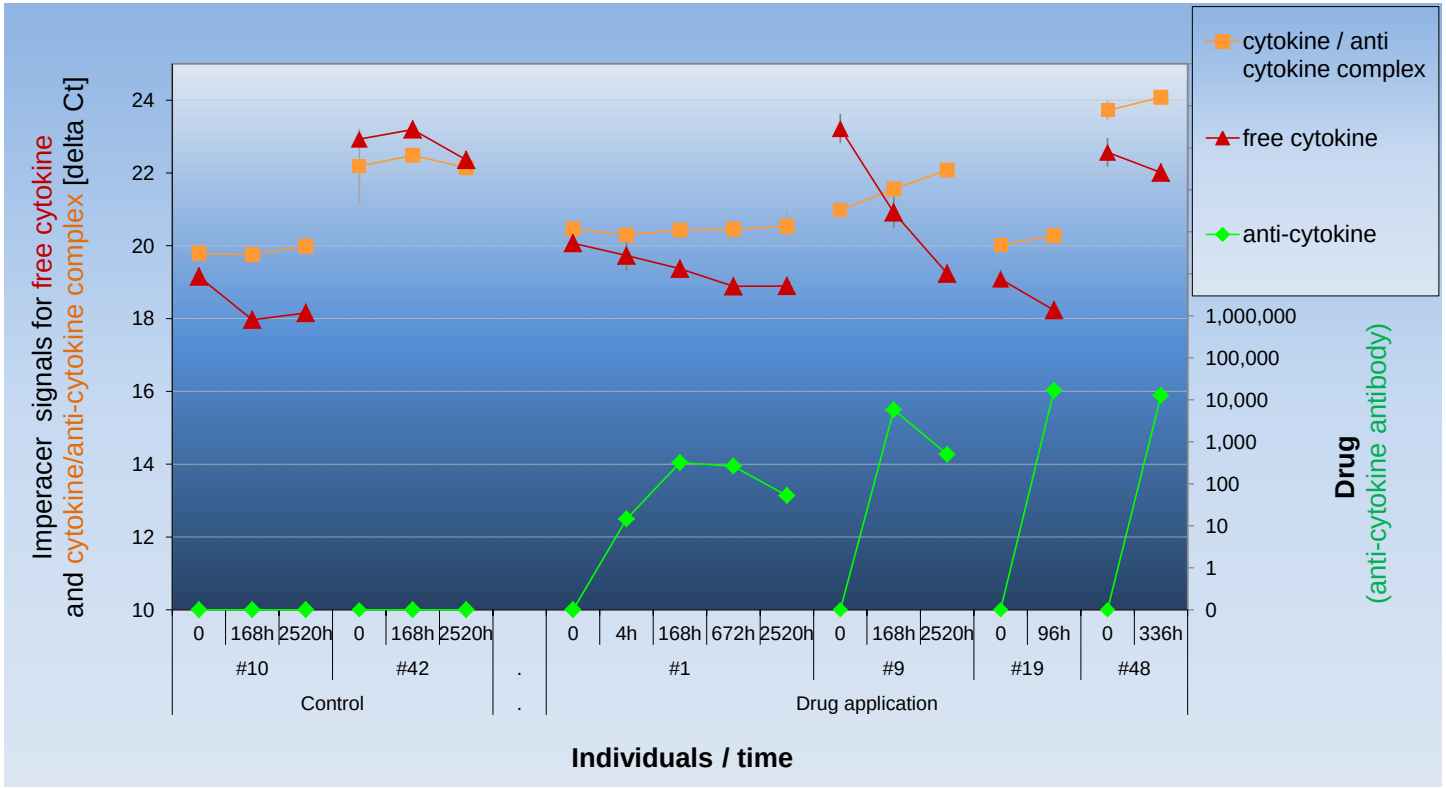
One sample → Multiple assays

Due to high sensitivity, sample dilution and small required assay volume, several parameters can be detected from a single sample in *polyplex* multiple determination for quantitative analysis. The parallel analysis in separate assays minimizes unspecifc interaction of reagents (in contrast to simultaneous multiplex analysis).

Target 6: Polyplex







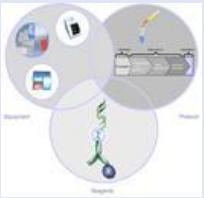
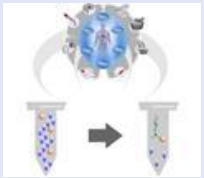
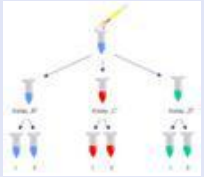
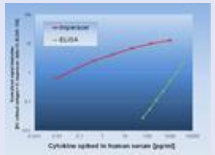
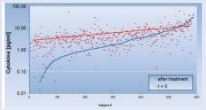
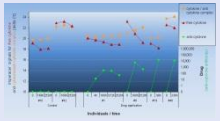
Clinical Study: Anti-Inflammatory Anti-Cytokine Drug (Therapeuthic Antibody)

Parallel analysis of anti-cytokine antibody („***drug***“), ***free cytokine*** and ***antibody-cytokine complex*** confirms the formation of cytokin / anti-cytokine binding after application of the drug.

The signal for the detectable amount of native free cytokine is decreased while the signal for in-vivo formed complex increases. While the amount of free cytokine varies for each individual and also over time, this typical „V“-pattern of complex increase and cytokine decrease allows to identify and monitor drug functionality.

Summary
&
Conclusion

Summary & Conclusions

- When monitoring the interaction between cytokines and cytokine-binding antibodies, there are different targets, such as free & bound cytokine or antibody-antigen complex.
- The Imperacer® protocol for Immuno-PCR (direct antibody-DNA conjugates, standardized reagents & real-time detection) allows for sensitive and robust detection of various targets in small sample sizes.
- Due to high sensitivity, the analysis of multiple compounds can be combined to get more information out of a single sample.
- By utilization of appropriate targets / antibodies, diesase and therapy research, diagnosis & monitoring can be significantly improved.

Contact information

Sensitivity by combination: immuno-PCR and related technologies

Michael Adler, Ron Wacker and Christof M. Niemeyer, Analyst, 2008, 133, 702

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