Enhancing assay sensitivity by incorporating signal amplification into a microfluidic assay

Linda Klauss, PhD, Senior Field Application Scientist

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What if higher sensitivity is needed?

Presentation outline

1 I Binding Oligo Ladder Detection (BOLD) amplification

2 I Biomarker assay development - BOLD

- TNFa
- NfL
- IL-4

3 I Summary and conclusions

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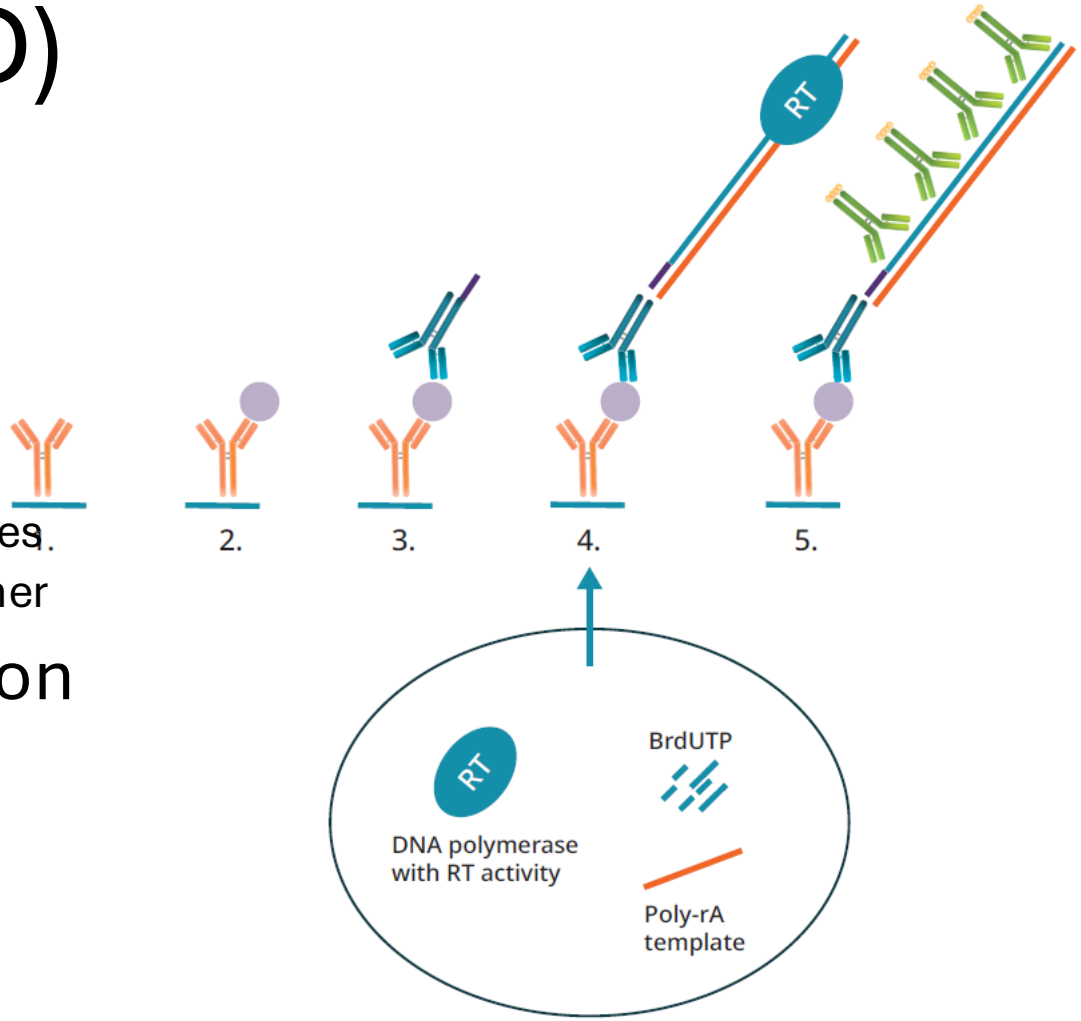
Signal amplification using Binding Oligo Ladder Detection (BOLD)

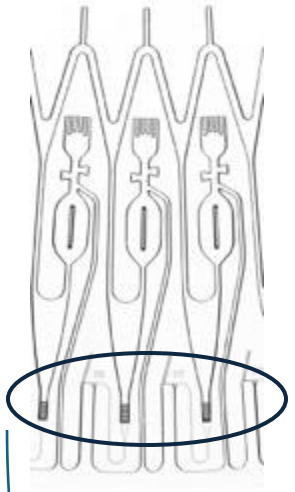
Signal amplification using Binding Oligo Ladder Detection (BOLD)

1. Capture reagent
2. Analyte
3. Detection reagent
    - Conjugated prior to running assay
        - Click-chem activation of amine-residues
        - Conjugation with DBCO – oligo-dT primer
4. DNA/RNA hybrid strand generation
    - Poly-rA template
    - Reverse transcriptase
    - BrdUTP
5. Detection Antibody – anti-BrdU

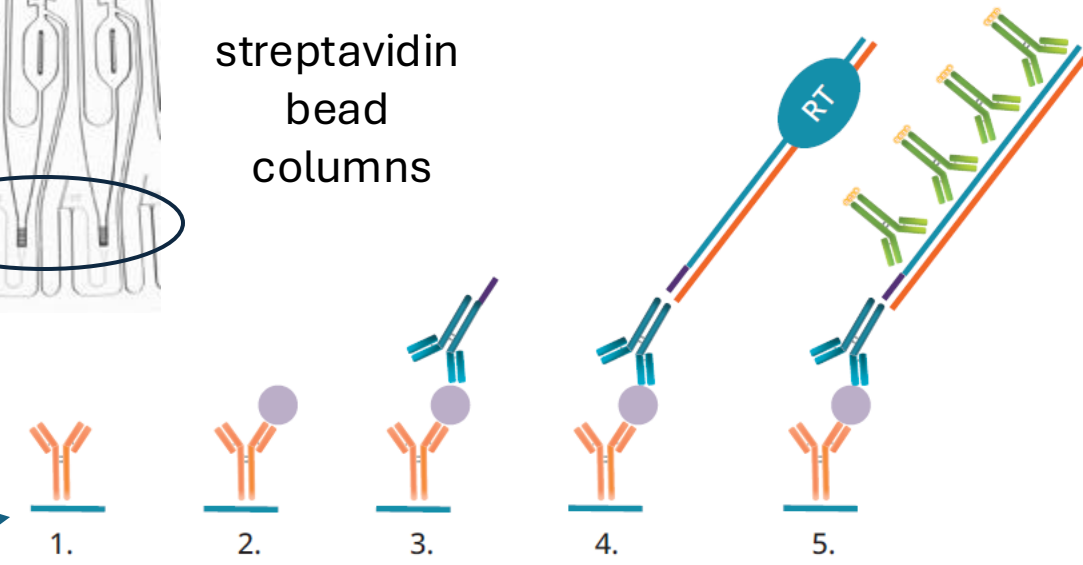


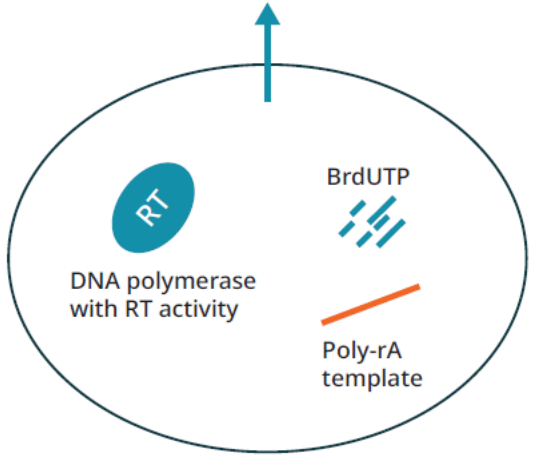
Signal amplification using BOLD in a microfluidic disk

1. Biotinylated capture reagent
2. Analyte
3. Detection reagent
    - Conjugated prior to running assay
        - Click-chem activation of amine-residues
        - Conjugation with DBCO – oligo-dT primer
4. DNA/RNA hybrid strand generation
    - Poly-rA template
    - Reverse transcriptase
    - BrdUTP
5. Detection Ab anti-BrdU - Alexa Fluor 647



streptavidin bead columns





Biomarker assay development using BOLD

Three biomarkers selected for proof-of-concept studies

Tumor necrosis factor alpha (TNFa)

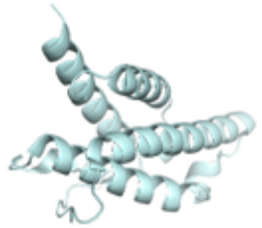
- Pro-inflammatory cytokine
- Low pg/mL baseline serum levels in healthy individuals
- Reagents based on ready-to-use cytokine kit

Neurofilament light chain (NfL)

- Biomarker for neuronal damage
- High levels in cerebrospinal fluid (CSF), low pg/mL levels in serum
- Exploratory assay developed

Interleukin 4 (IL-4)

- Pleiotropic cytokine
- Low pg/mL baseline levels in healthy individuals
- Reagents based on ready-to-use cytokine kit

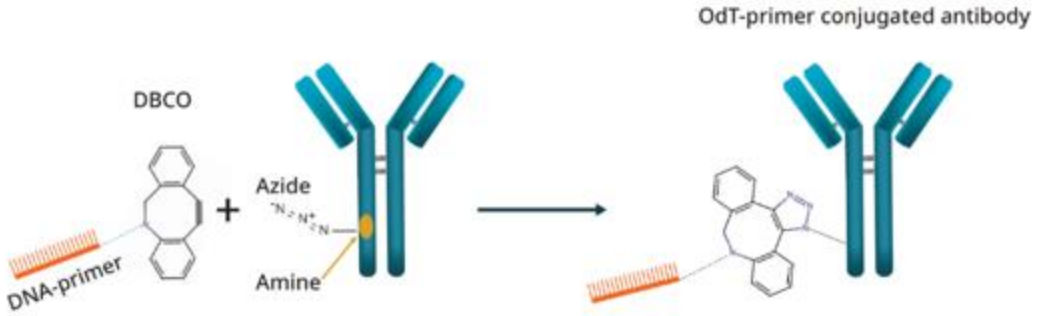


Protein databank entries 2TNF, 2B8U and AlphaFold model AF-P07196-F1-v6

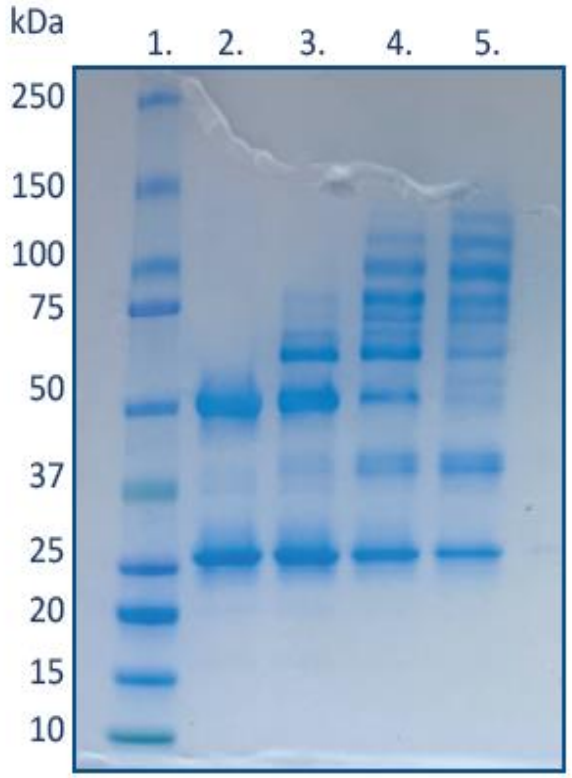
TNFa assay development

TNFa - conjugation of detection reagents

- Detection reagent from TNFa kit (unlabeled)
- Conjugation conditions with varying molar excess off:
    - Azide to antibody
    - DBCO-linked primer to azide-coupled antibody



DBCO - dibenzocyclooctyne



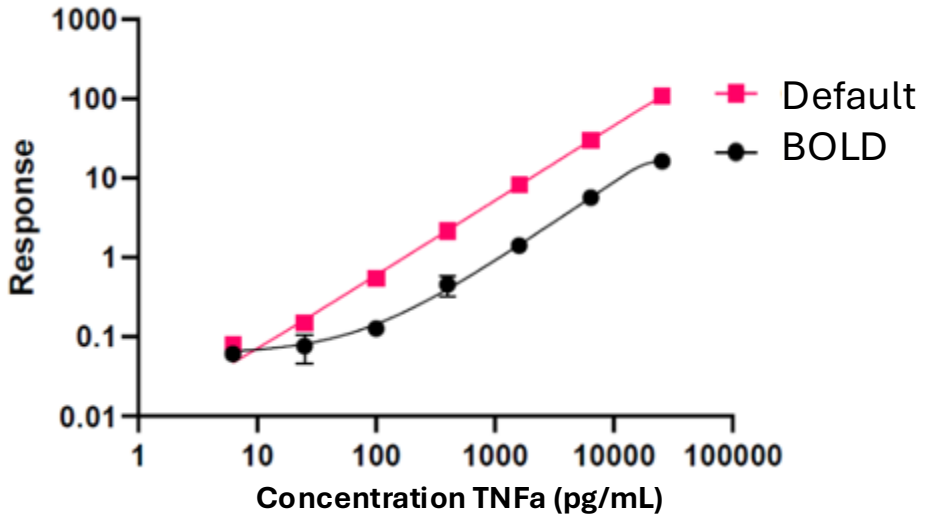
1. Protein standard
2. Unmodified antibody
3. Conjugate 5x:5x
4. Conjugate 20x:20x
5. Conjugate 40x:40x

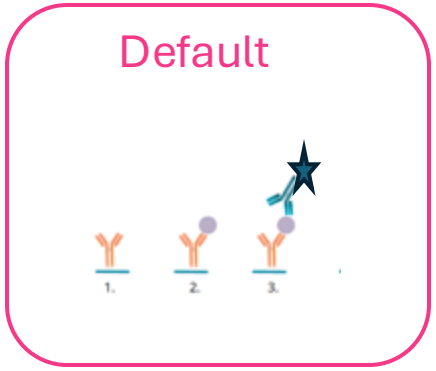
SDS-PAGE analysis of anti-TNFa:oligo-dT primer conjugates

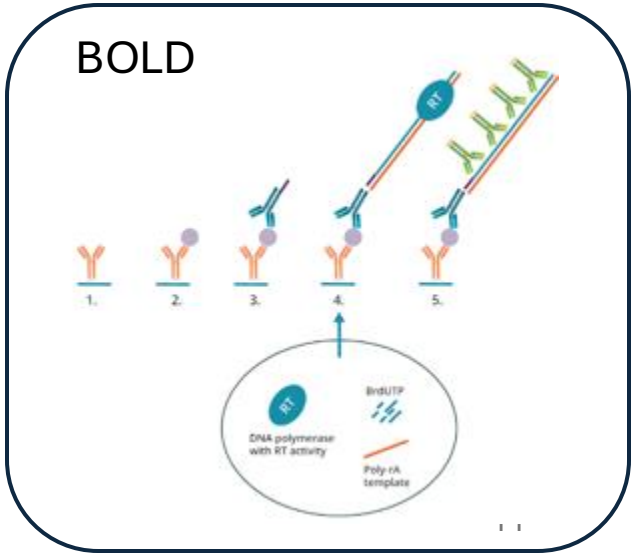
TNFa – initial test

- BOLD principle works on the column of a microfluidic disc
  - DNA / RNA hybridization takes place
  - Polymerase is active

- Optimization required
  - Default assay outperforms amplified method



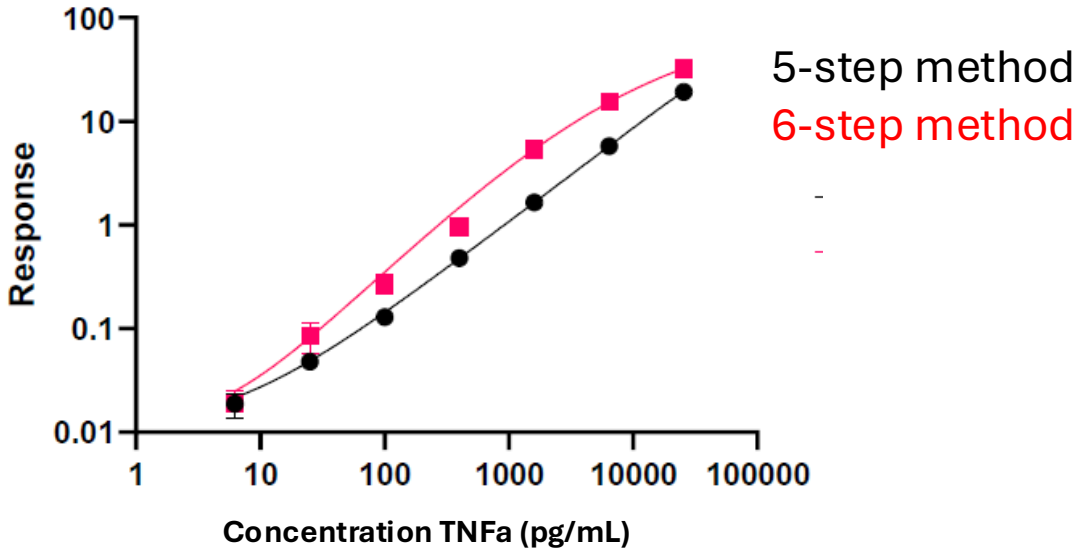


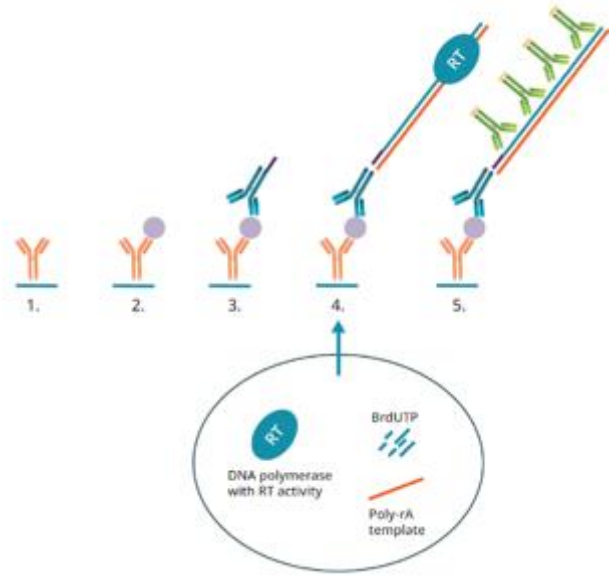


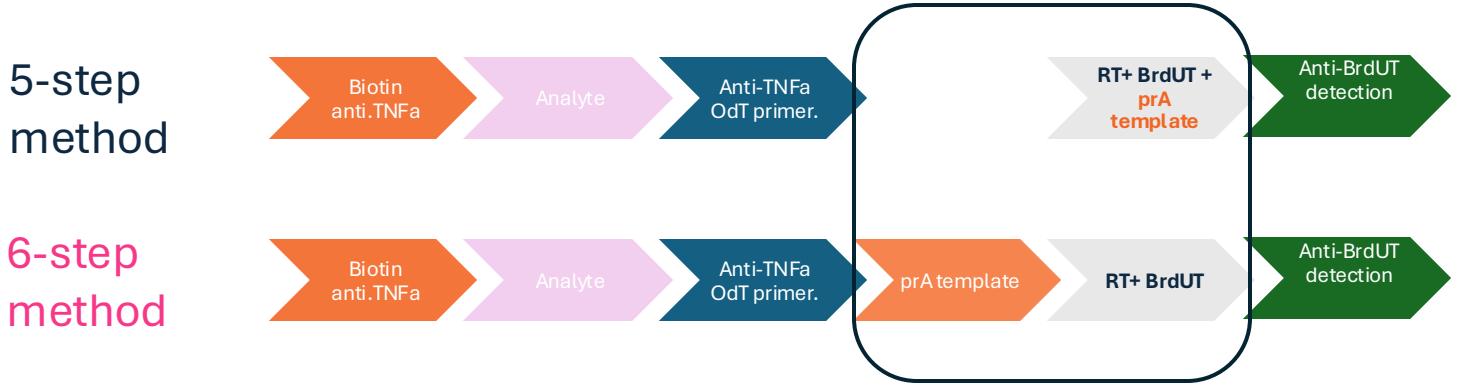
TNFa – separation of annealing and amplification

- Small improvement in assay performance when prA template is added prior to RT reaction mixture

- 6-step method used for all fur

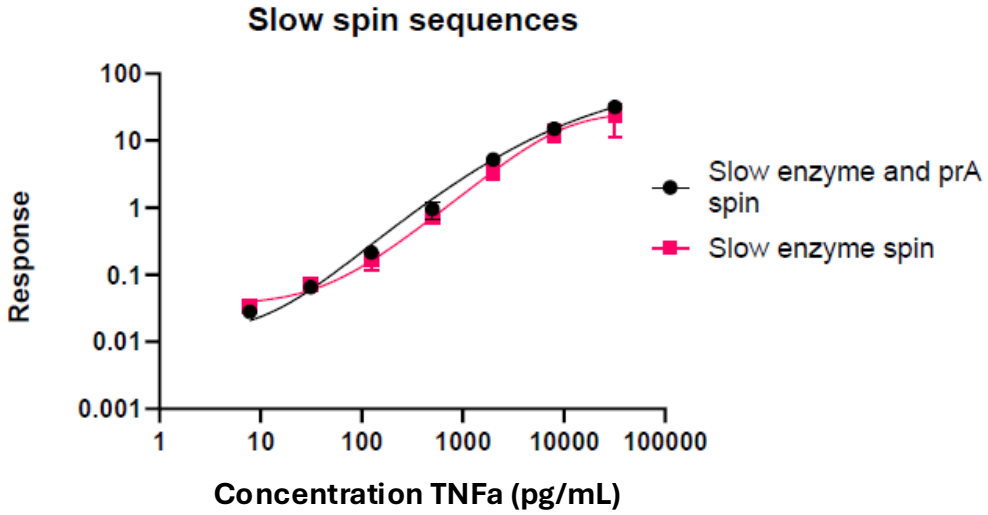


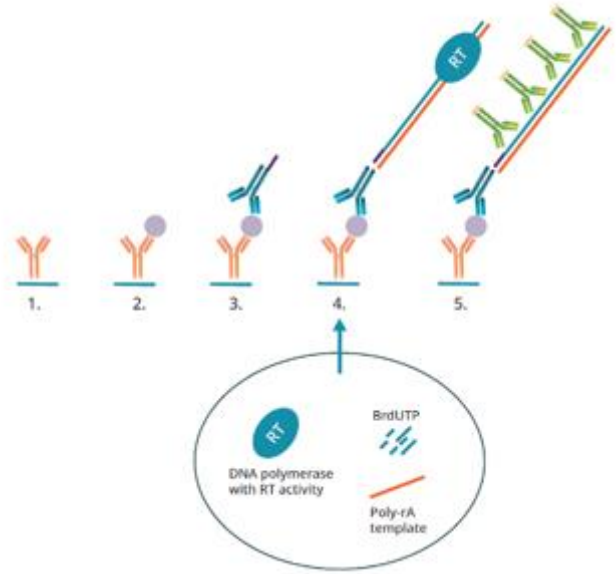




TNFa – exploring methods

- Implementation of slow spin methods to increase contact time with column
  - Minor improvement





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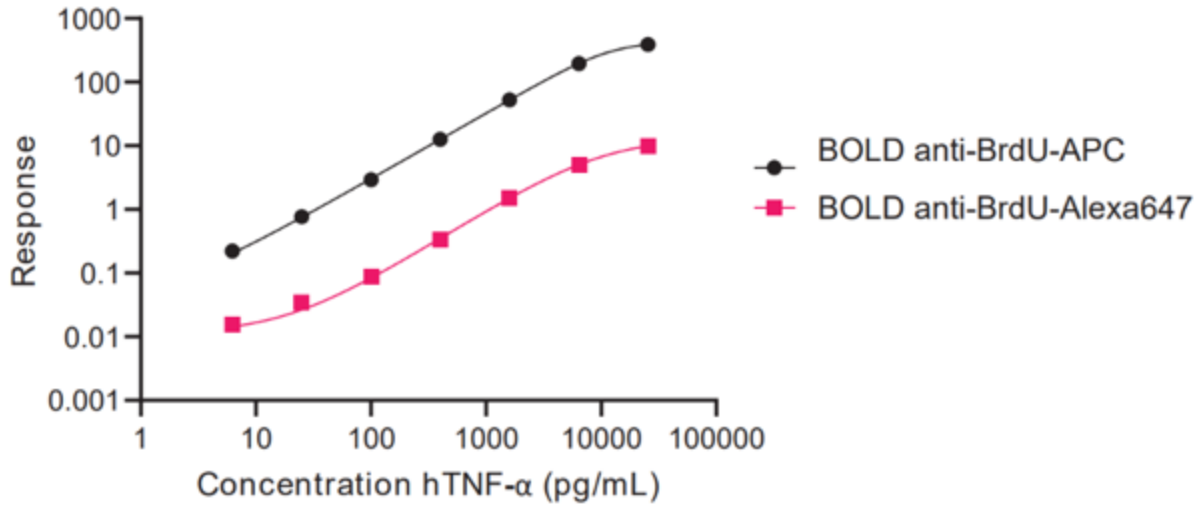
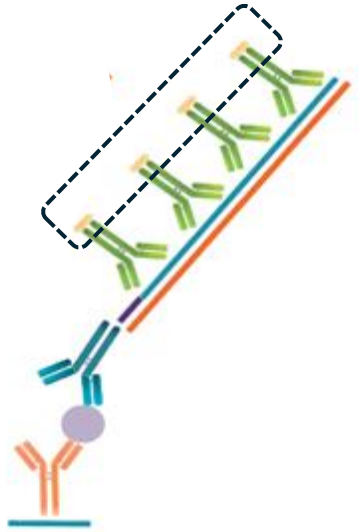
TNFa - alternative fluorophore detection reagent improves sensitivity

- Hypothesis that the Alexa Fluor 647 labeling damaged the detection antibody
  - Known challenge that this antibody is difficult to label without losing affinity
  - APC-label outperforms Alexa Fluor 647 for this dy





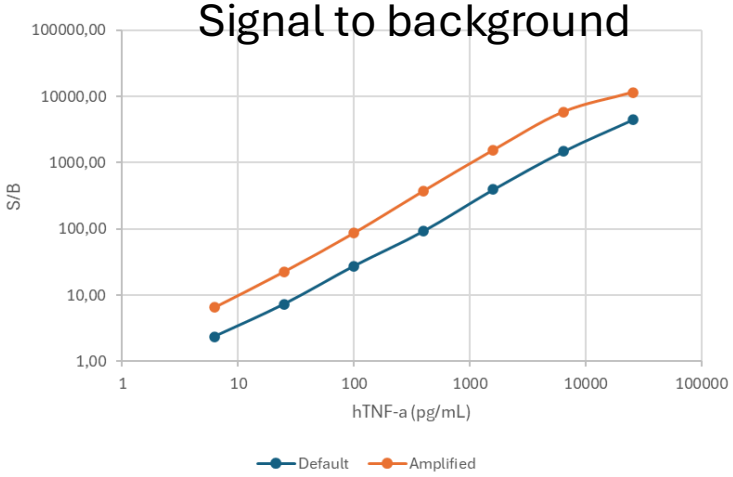
TNFa – final conditions

- Amplified method is now more sensitive compared to default method (TNFa kit)
  - Signal to background improves with a factor 3 over the whole range

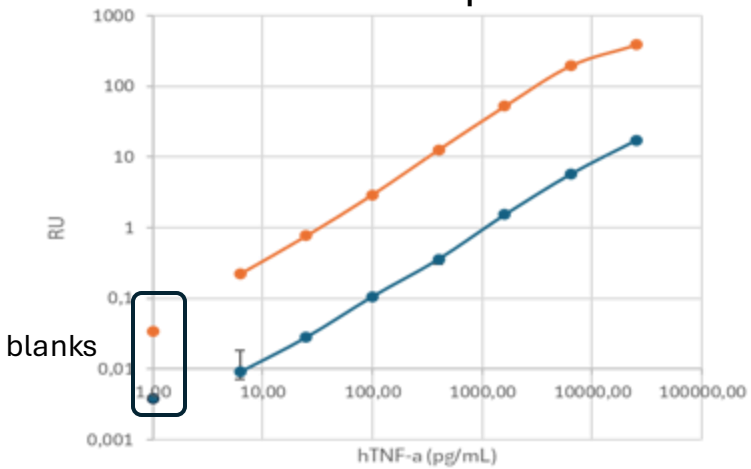
Assay	LOD (pg/mL)	LLOQ
TNFa default	< 0.7	1 pg/mL (in well) 2 pg/ml (neat)
TNFa amplified	< 0.039	tbd

- Initial assay conditions and methods identified for further exploration

Signal to background



Absolute response



Bioaffy 4000 CD

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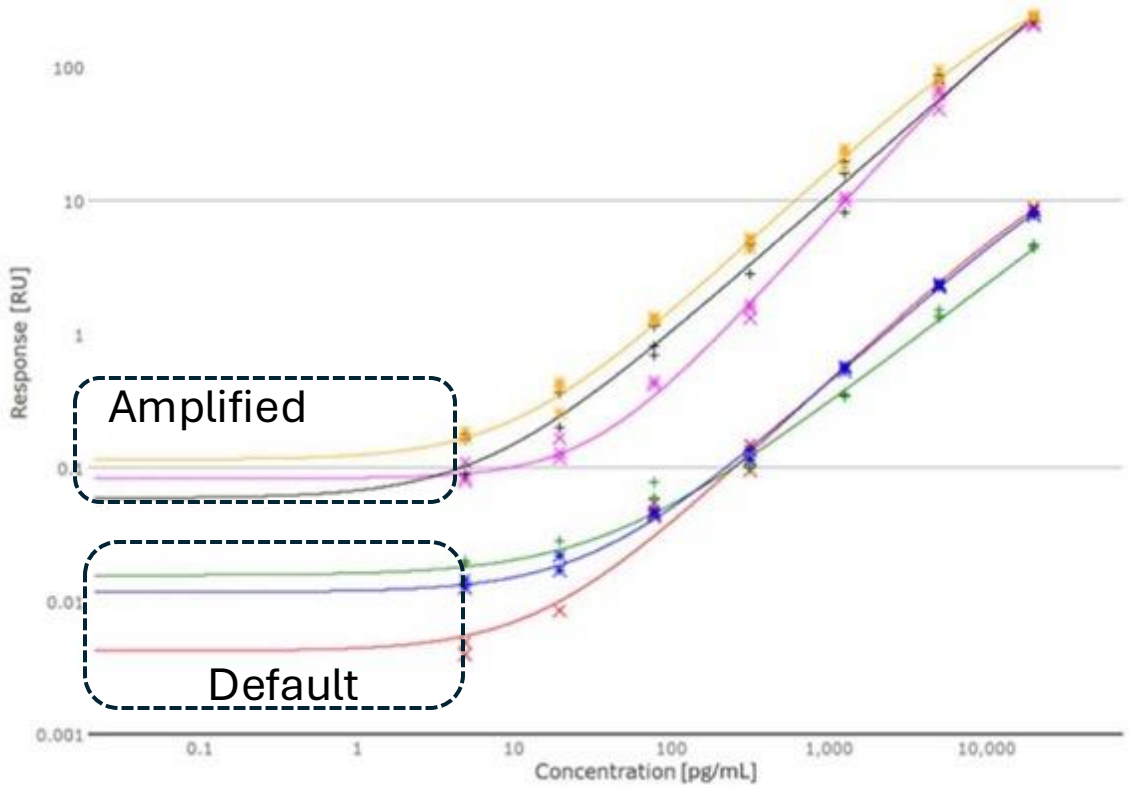
NfL assay development

NfL - reagent screen and conjugation of detection antibody

- Reagent screen
  - Reagent combinations were tested in standard assay
  - Promising detection reagents were conjugated to be used in BOLD assay

- Conjugation conditions
  - 20 times molar excess azide to antibody
  - 5 times molar excess DBCO-primer to azide activated antibody

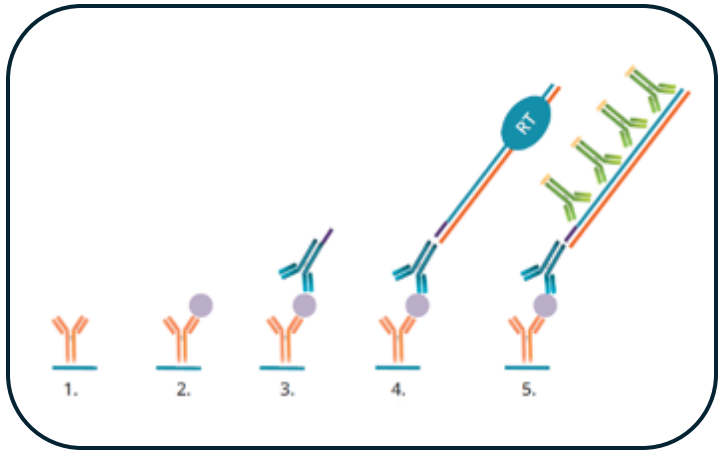
NfL reagent screen - minor effect of using amplification method

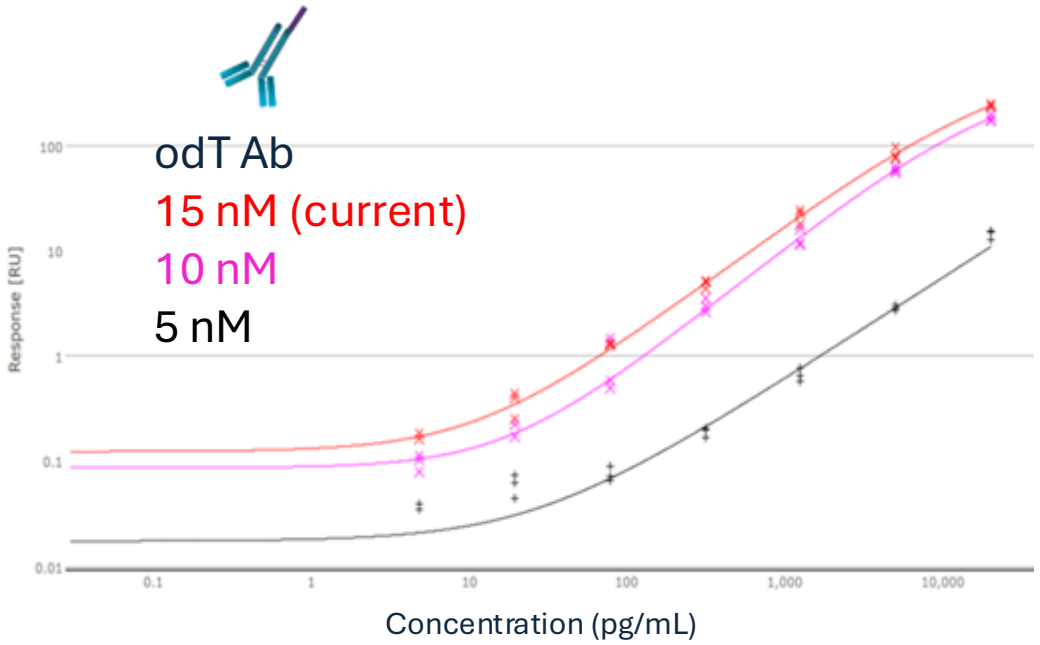


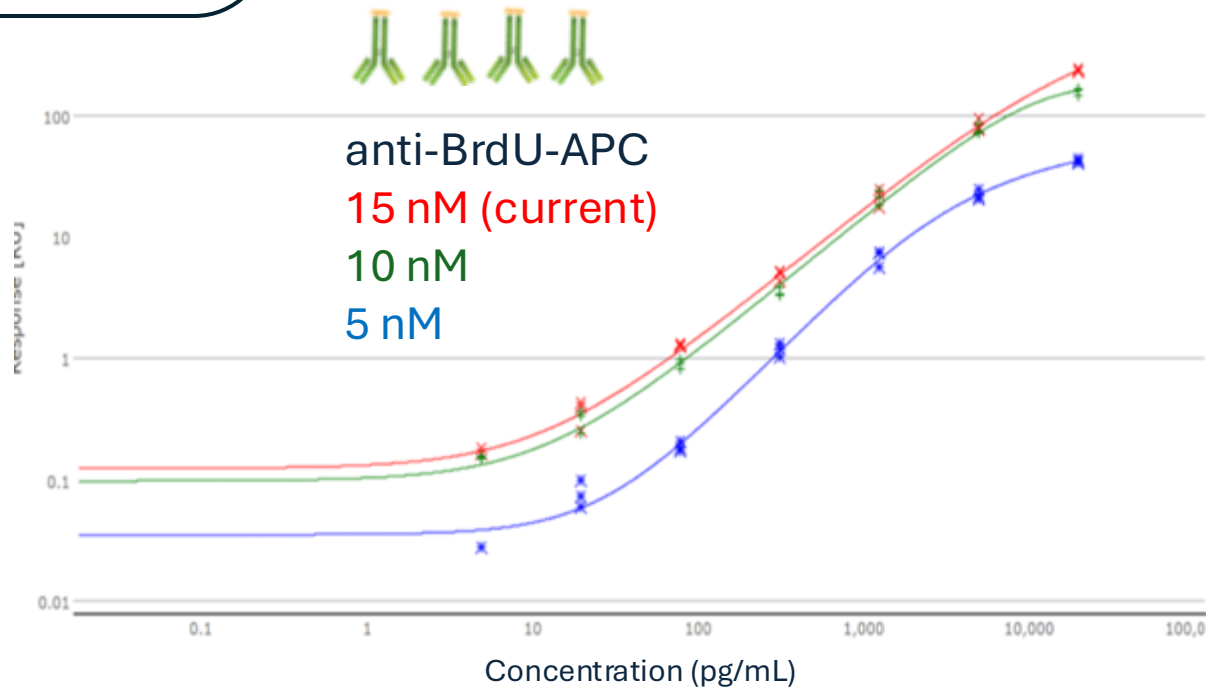
Reagent	Det A	Det B	Det C
Cap C	Default x Amplified X		
Cap D		Default + Amplified +	Default * Amplified *

- Reagent pair capture D / detection C was explored further
  - Aim to bring background down while not losing specific signal

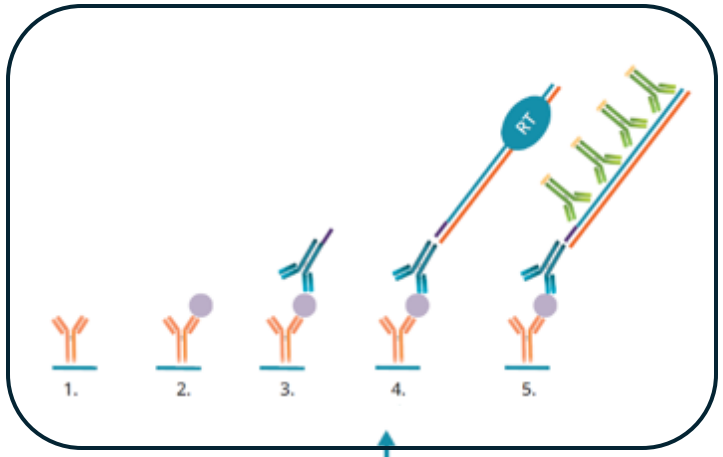
NfL assay optimization - current conditions work best

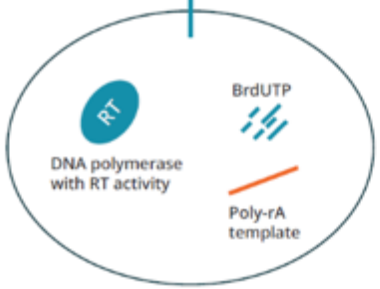




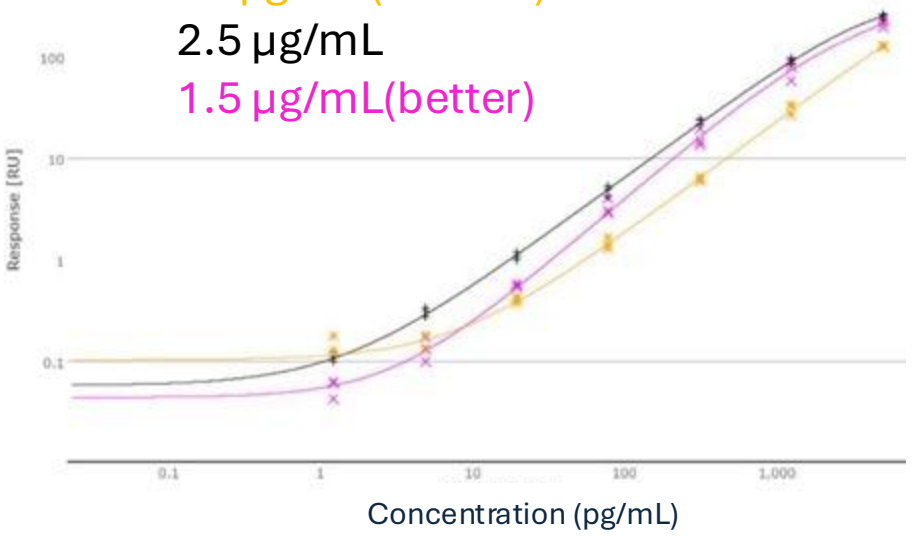


NfL - lowering template concentration improves assay sensitivity

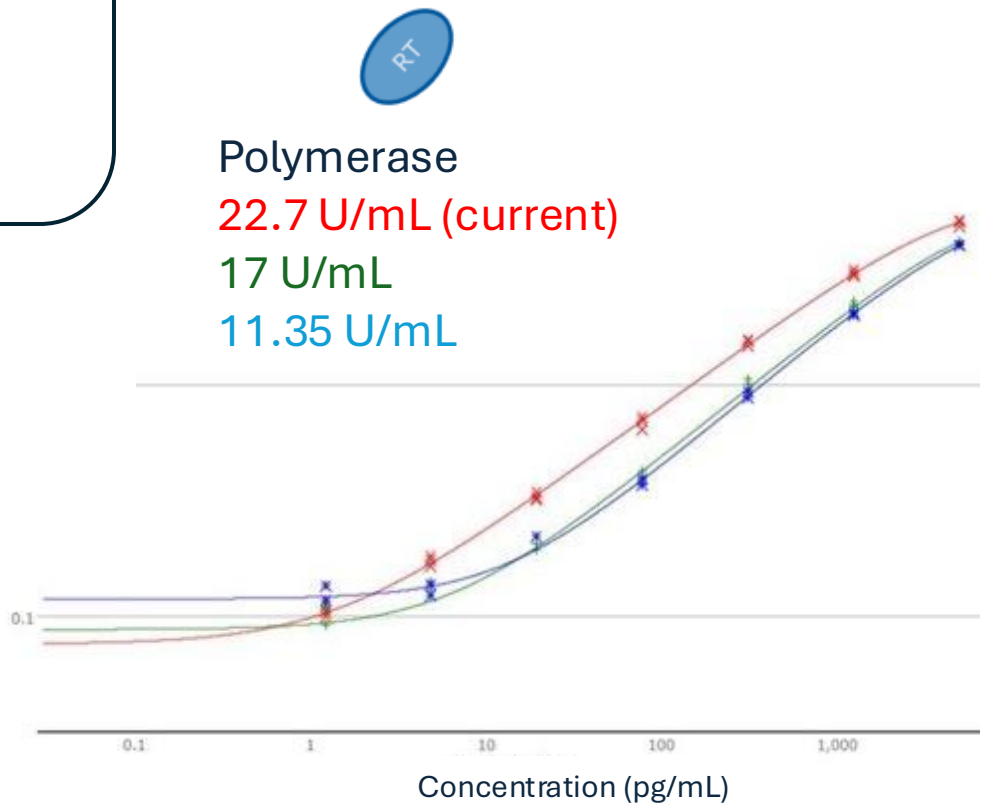




Template
10 µg/mL(current)
2.5 µg/mL
1.5 µg/mL(better)

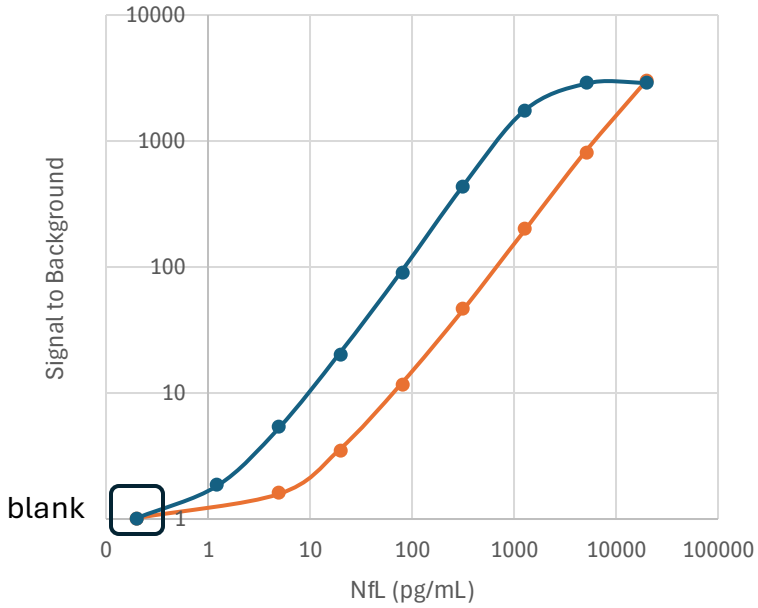


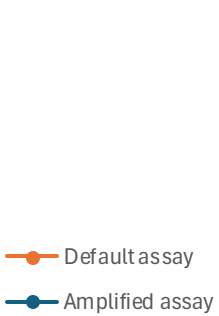
Polymerase
22.7 U/mL (current)
17 U/mL
11.35 U/mL

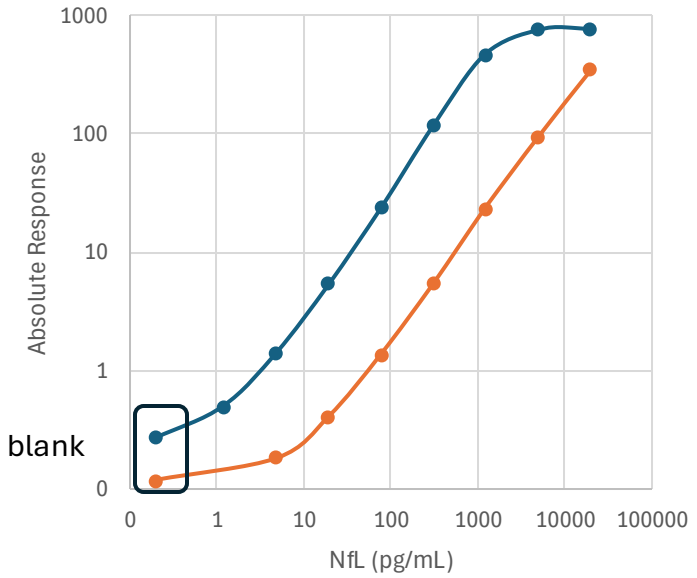


NfL - amplification significantly improves assay sensitivity

- Optimization of the assay conditions improved the sensitivity of the assay
  - Signal to background improves up to a factor 10
  - Lowering the template concentration was key





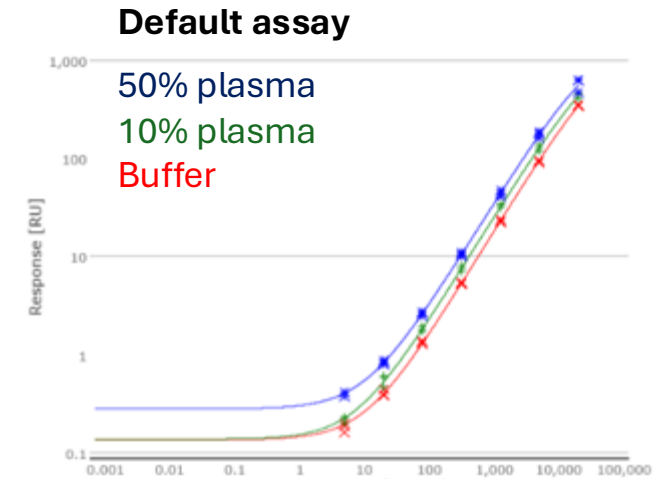


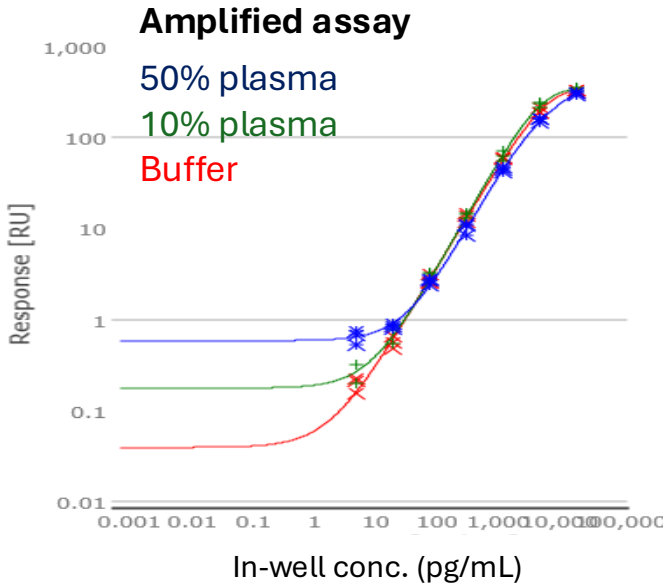
NfL – preliminary work with human matrix

- Standard curve was prepared in buffer containing 10% or 50% human serum pool

- Endogenous levels can be detected
  - Raised background likely reflects endogenous levels
    - Back-calculated corresponds to ± 25-65 pg/mL
    - Same experiment in cerebrospinal fluid (CSF) gave > 30x higher values

- Next steps
  - Sample dilutional linearity / parallelism
  - Bridging



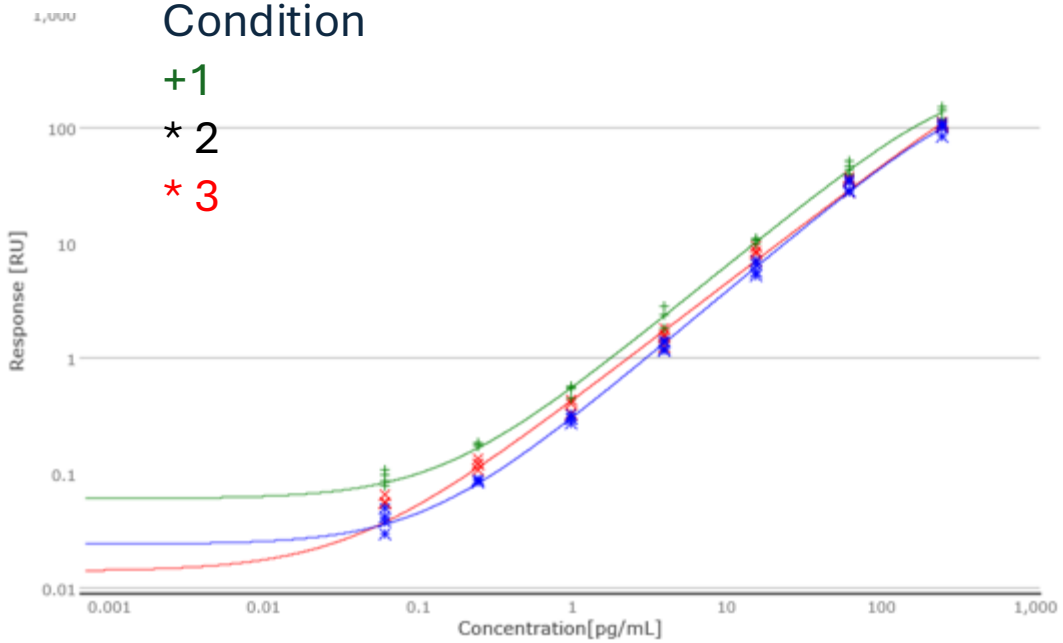


IL-4 assay development

IL-4 - exploring conjugation protocols for odT detection reagent

- Detection reagent from IL-4 kit was conjugated using different conjugation protocols
  - Condition 3 works best in this case
    - Lowest background
    - Highest S/B

Molar excess Condition	Step 1 activation	Step 2 odT primer
1	Click-chem 20x	30x
2	Click-chem 20x	5x
3	Site specific click-chem	30x



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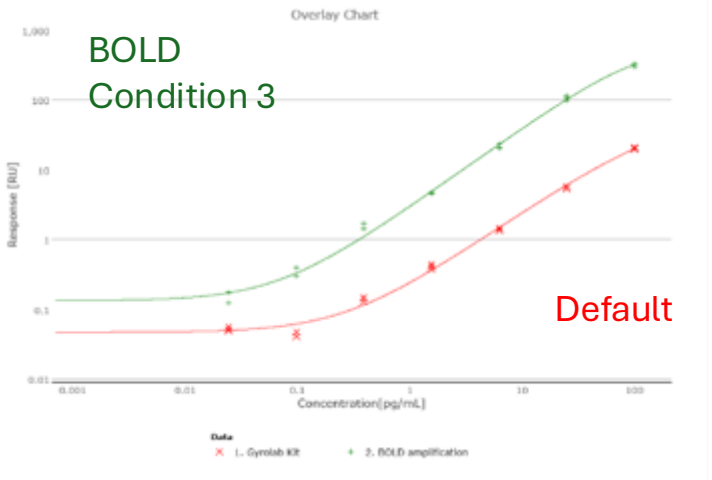
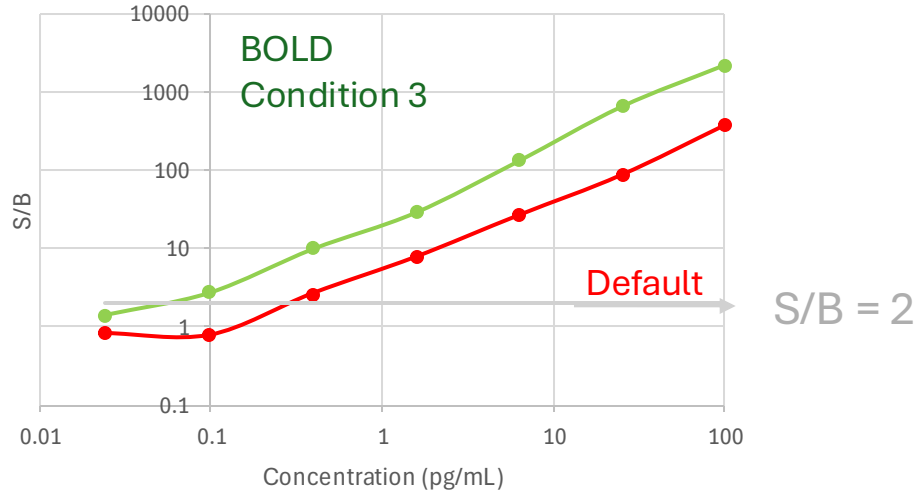
IL-4 example – best BOLD conditions compared to default assay

- Small improvement in sensitivity for BOLD assay
  - Factor 3 – 5 improvement in signal to backgrour
  - Template condition not optimised yet



Assay	LOD (pg/mL)	LLOQ (pg/mL)
IL-4 default	< 0.5	0.8 (in well) 1.6 (neat)
IL-4 BOLD Condition 3	< 0.08 – 0.2	Tbd



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Summary and conclusions

Conclusions

- Preliminary work shows that BOLD reagents are compatible with the microfluidic discs at room temperature
- Improvement of signal over background compared to default assay (factor 3 – 10) for three different biomarker assays
- Possible additional optimizations
  - Buffers / method washes / conjugation protocols for odT antibody
- Next steps
  - In depth characterization of BOLD assays and measuring samples!

Acknowledgements

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Cavidi

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- Peter Stenlund
- Johanna Hedin

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QUESTIONS