# Neutralizing anti-drug antibodies

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# FDA says:

*Because of the size of some clinical trials and the necessity of testing patient samples at several time-points, FDA recommends a multi-tiered approach to the testing of patient samples. ….*

*Neutralizing antibodies (NAB) are generally of more concern than binding antibodies (BAB) that are not neutralizing, but both may have clinical consequences.*

# Question

Why are neutralizing antibodies (NAB) of more concern than binding antibodies (BAB) that are not neutralizing if both may have clinical consequences?

Do NAB assays more realistically reflect the situation in the body?

# FDA says:

*Generally, bioassays have significant variability and a limited dynamic range for their activity curves. Such problems can make development and validation of neutralization assays difficult and FDA understands such difficulties. Nonetheless, we will recommend such assays because they are critical to understanding the importance of patient immune responses to therapeutic proteins.*

# Requirements for cellular assays

- Suitable cell line
- Linearity
- Interference
- Cut point
- Sensitivity
- Specificity
- Precision
- Robustness
- Ruggedness

# NAB analysis

- Cell based assays (CBA)
  - Proliferation
  - Gene expression
  - Gene reporter
  - Signal transduction
- Competitive ligand binding assays (CLBA)
  - ECL
  - Biacore

# Example: Erythropoietin

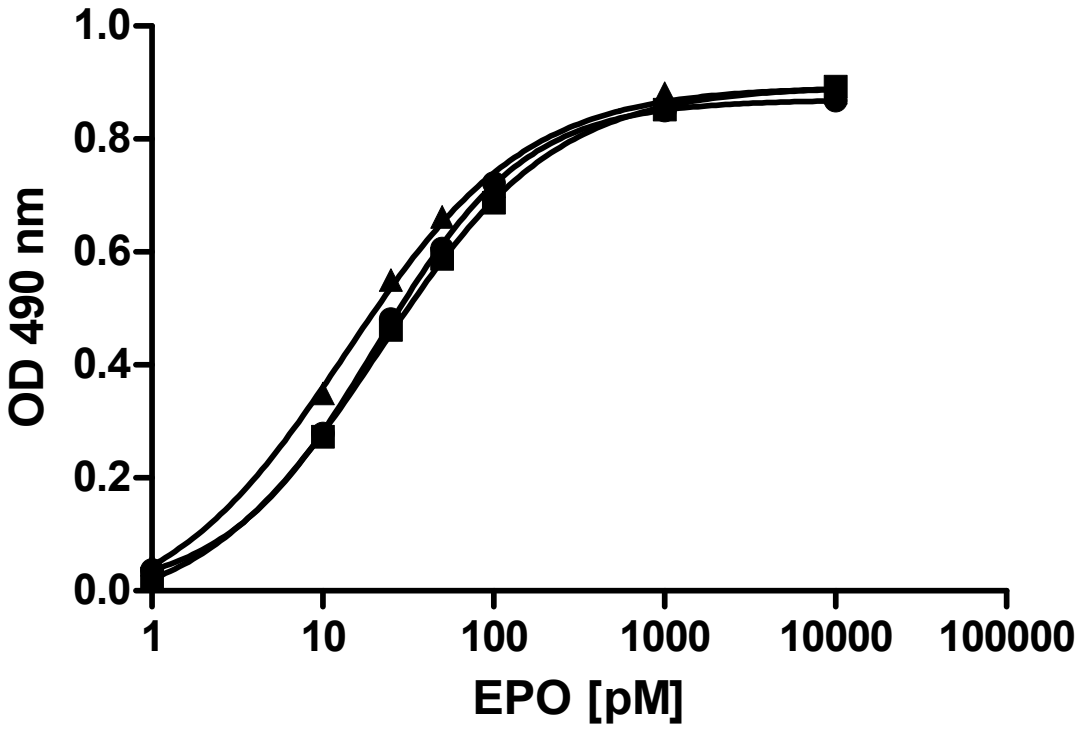
a recombinant human protein drug with a non-redundant endogenous counterpart

used for the treatment of renal and non-renal anemia

# Antibodies against EPO

1. Pure red cell aplasia (PRCA) after initial successful erythropoietin therapy
   - Progressive, transfusion-dependent anemia
   - Almost total lost of erythroid progenitor cells with normal BM
2. Antibodies against erythropoietin
3. No endogenous erythropoietin detectable

# EC50 Determination




EC50 (pM)

- Exp.1/0%: 21
- Exp.2/0%: 21
- Exp.3/0%: 14

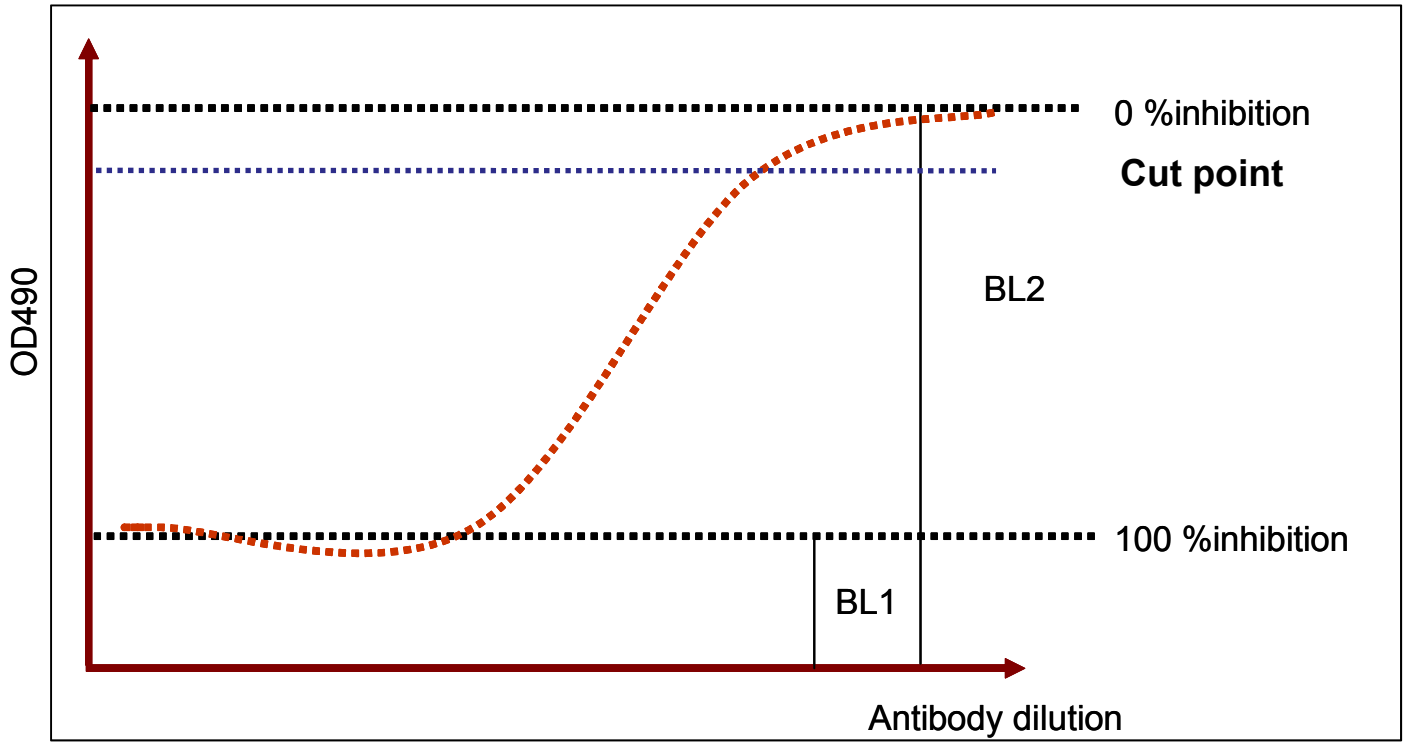
EPO receptor expressing cell line (UT7, TF1)

# Interference w/ IL-3

| | IL-3 [pg/mL] | | | | |
|---|---|---|---|---|---|
| | **0** | **50** | **100** | **500** | **1,000** |
| % inhib | 95 | 96 | 94 | 82 | 68 |
| Diff [%] | 13% | 14% | 10% | 14% | 13% |
| AC | ≤ 30% | ≤ 30% | ≤ 30% | ≤ 30% | ≤ 30% |

There is no interaction with IL-3 leading to a significant change of inhibitory effect of anti-EPO antibodies. Average IL-3 level in normal healthy subjects is 27 pg/ml

# Cut point




Mean inhibition + 3.09 SD of NHC: 17 % inhibition

# Sensitivity/LLOD

| Monkey anti-EPO (ng/ml) | %-Inhibition | | | | SD | %CV | AC |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | mean | | | |
| **200** | 50.1 | 52.2 | 55.3 | 52.5 | 2.1 | 4.1 | ≤ 30% |
| **100** | 71.9 | 70.6 | 71.9 | 71.5 | 0.6 | 0.9 | ≤ 30% |
| **50** | 48.5 | 52.7 | 56.8 | 52.7 | 3.4 | 6.4 | ≤ 30% |
| **25** | 21.0 | 17.1 | 28.3 | 22.2 | 4.6 | 20.9 | ≤ 30% |
| **12.5** | 7.3 | 6.0 | 13.0 | 8.7 | 3.0 | 34.8 | ≤ 30% |
| **6.25** | 6.5 | 0.3 | 7.3 | 4.7 | 3.1 | 67.1 | ≤ 30% |
| **3.13** | 3.9 | 6.2 | 4.9 | 5.0 | 1.0 | 19.0 | ≤ 30% |
| **1.55** | -0.1 | 4.2 | 8.0 | 3.8 | 3.6 | 94.9 | ≤ 30% |
| **0** | -5.2 | 2.4 | 2.9 | 0.0 | 3.7 | - | ≤ 30% |

# Precision

Intra-Assay

| | 1 | 2 | 3 | 4 | mean | SD | %CV | AC |
|---|---|---|---|---|---|---|---|---|
| **PC1** | 98.6 | 95.1 | 100.8 | 98.0 | **98.1** | 2.6 | 2.1 | ≤ 30% |
| **PC2** | 37.2 | 35.5 | 37.7 | 37.1 | **36.9** | 0.8 | 2.6 | ≤ 30% |

Inter-Assay

| | 1 | 2 | 3 | mean | SD | %CV | AC |
|---|---|---|---|---|---|---|---|
| **PC1** | 98 | 100 | 99 | **99** | 0.9 | 0.9 | ≤ 30% |
| **PC2** | 46 | 39 | 37 | **41** | 3.7 | 9.1 | ≤ 30% |

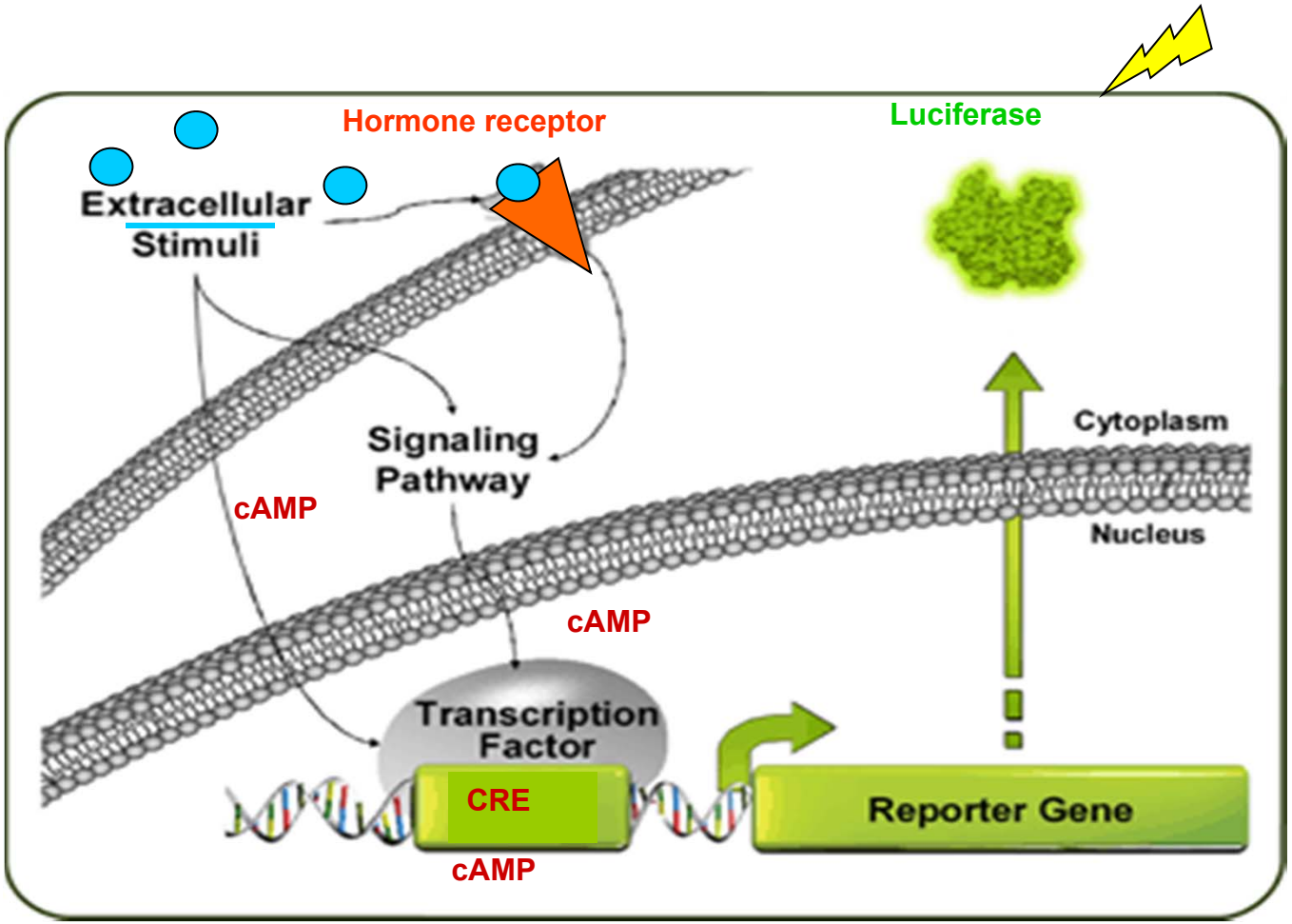
# Summary: NAB EPO

| Validation characteristics | Data |
|---|---|
| Challenging concentration of EPO | 20 pM |
| Intra-assay precision | ≤ 2 % CV |
| Inter-assay precision | ≤ 9 % CV |
| Stability for 3 days at +2-8°C | ≤ 8 % deviation |
| Stability for 3 weeks at -20°C | ≤ 12 % deviation |
| Stability at ≤ -15 °C after 3 Freeze/Thaw cycles | ≤ 11 % deviation |
| Stability at ≤ -70 °C after 3 Freeze/Thaw cycles | ≤ 14 % deviation |
| Drug tolerance | 250 mIU/ml |
| Clinical Specificity | 100 % |
| Cross reactivity against IL-3 | none |
| Screening cut point (% inhibition) | 17 % |
| Sensitivity in 2% serum | 25 ng/mL |
| Sensitivitiy in undiluted serum | 1250 ng/mL |
| Minimum required dilution (MRD) | 1 % serum |

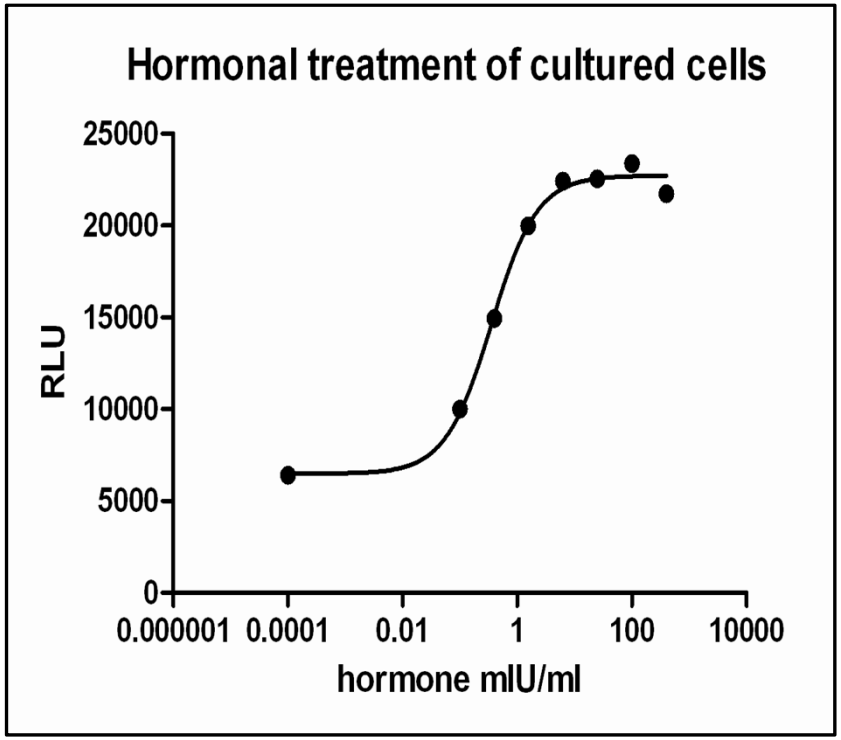
# Example: FSH

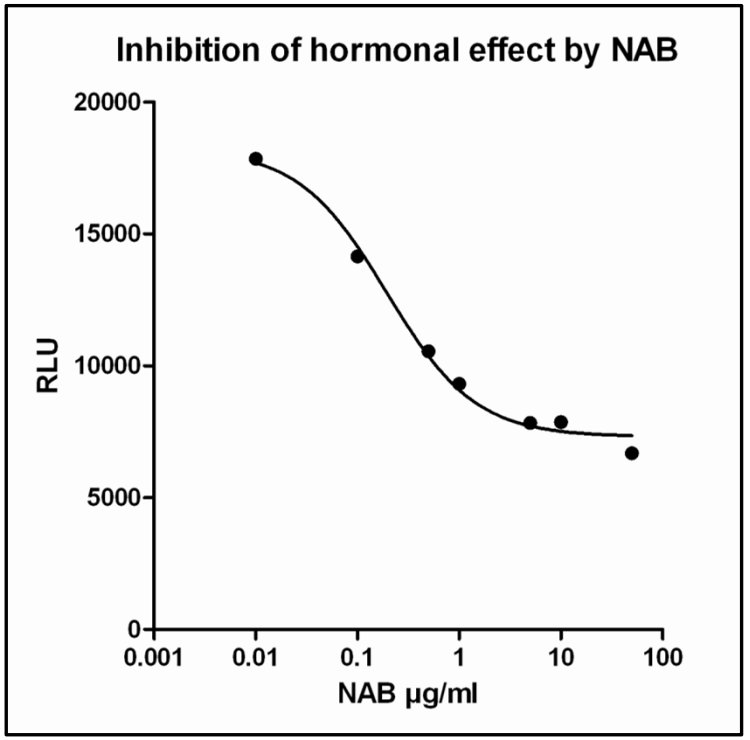
a recombinant human protein drug with an endogenous counterpart used for the treatment of induction of ovulation/pregnancy and for the development of multiple follicles.

# NAB against FSH



# NAB FSH


Hormonal treatment of cultured cells
RLU
25000
20000
15000
10000
5000
0
0.000001
0.0001
0.01
1
100
10000
hormone mIU/ml


Inhibition of hormonal effect by NAB
RLU
20000
15000
10000
5000
0
0.001
0.01
0.1
1
10
100
NAB µg/ml

# NAB FSH

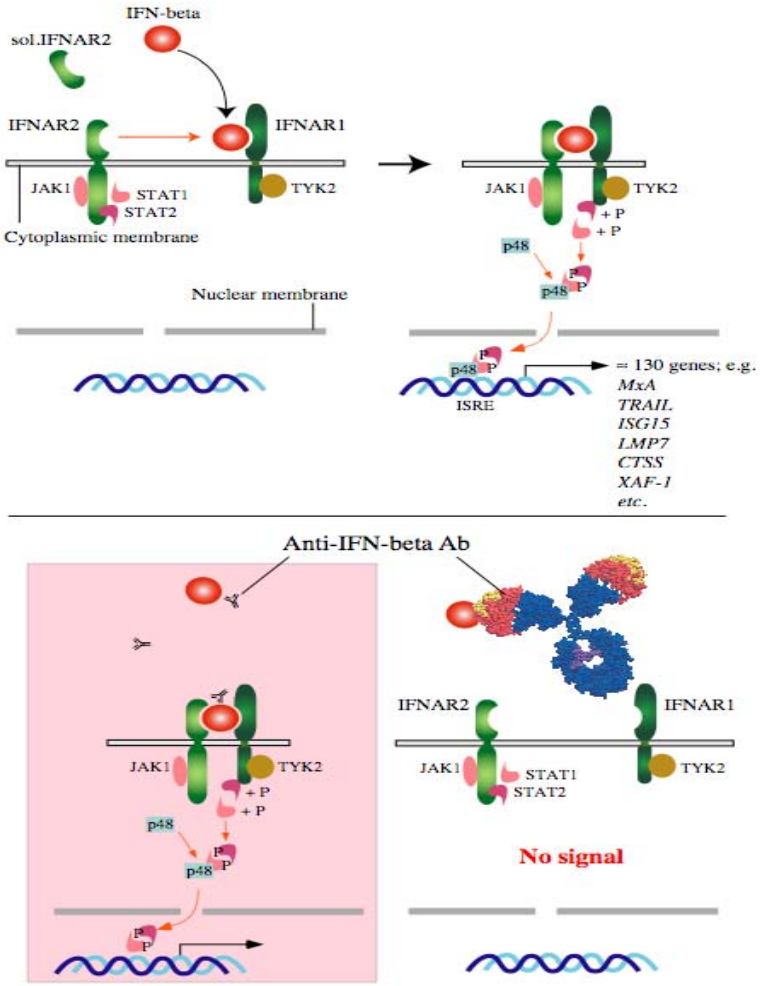
| Validation characteristics | Data |
|---|---|
| Challenging concentration of FSH | 1 mIU/ml |
| Intra-assay precision | ≤ 4 % CV |
| Inter-assay precision | ≤ 14 % CV |
| Stability for 3 days at RT | ≤ 11 % deviation |
| Stability for 3 days at +2-8°C | ≤ 5 % deviation |
| Stability at ≤ -15 °C after 3 Freeze/Thaw cycles | ≤ 11 % deviation |
| Stability at ≤ -70 °C after 3 Freeze/Thaw cycles | ≤ 14 % deviation |
| Drug tolerance at 150 µg/ml | 7.5 ng/mL |
| Drug tolerance at 15 µg/ml | 0.75 ng/mL |
| Clinical Specificity | 99 % |
| Cross reactivity against LH, TSH, CGalpha | None |
| Screening cut point (% inhibition) | 23 % inhibition |
| Sensitivity | 100 ng/ml |
| Minimum required dilution (MRD) | 2 % serum |

# Example: Interferon

a recombinant human protein drug with an endogenous counterpart used for the treatment of Multiple Scleroris (IFN-β) and Hepatitis virus infection (IFN-α)

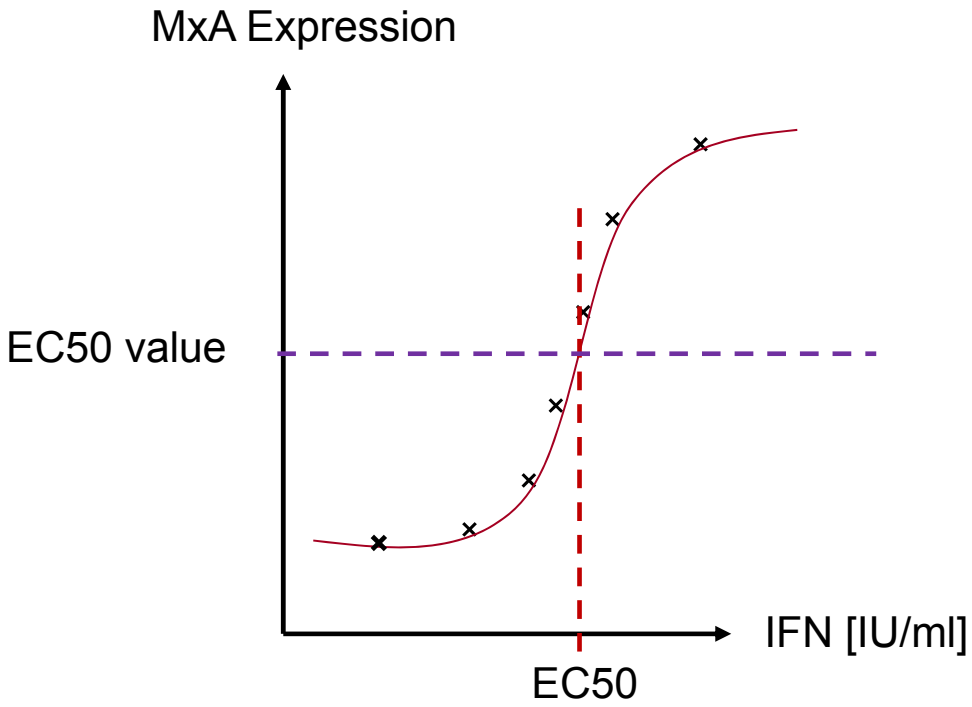
# Gene expression assay

## NAB against inferferon



# Gene expression assay

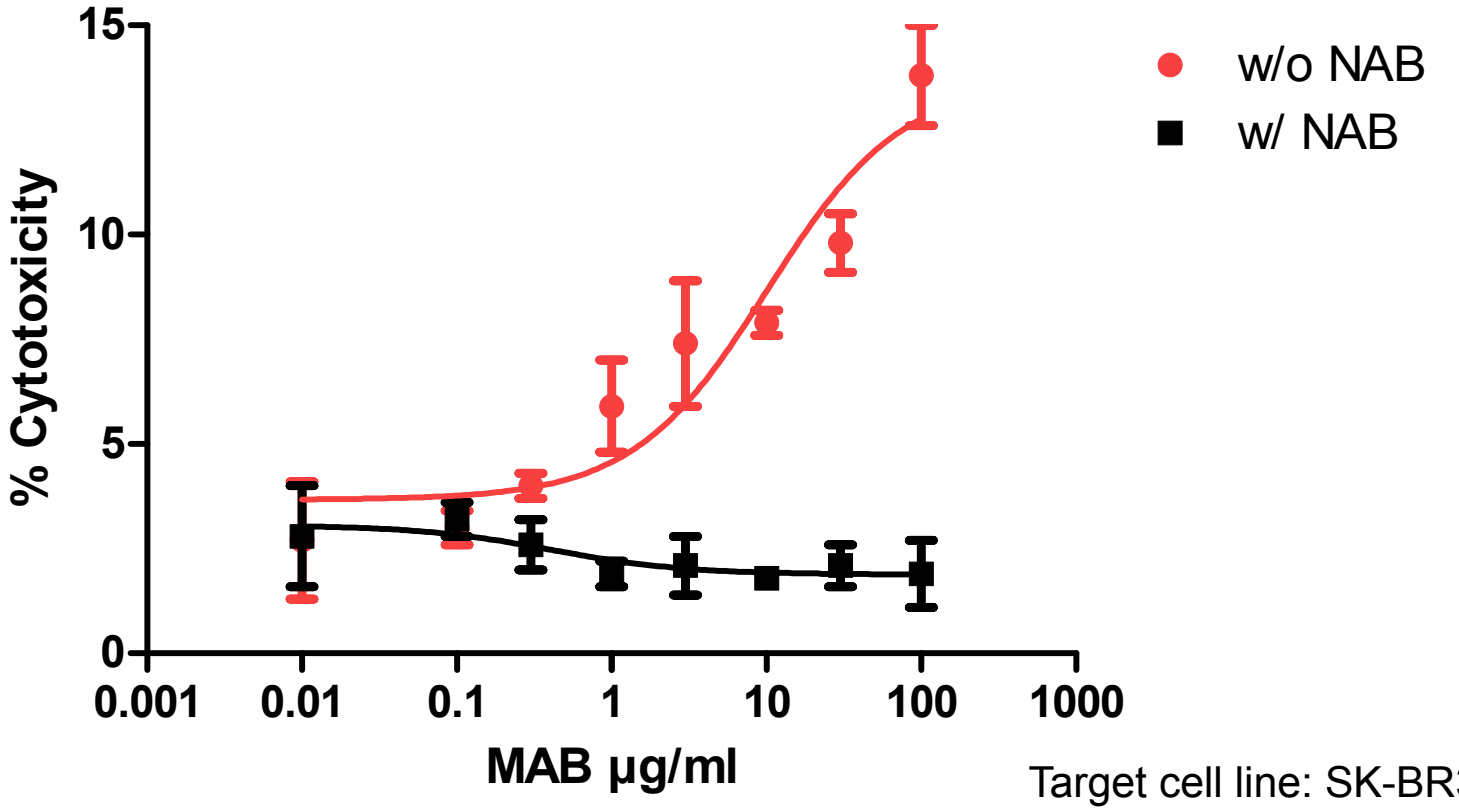
## NAB against inferferon by MxA analysis




**Positive** sample: sample signal < EC50

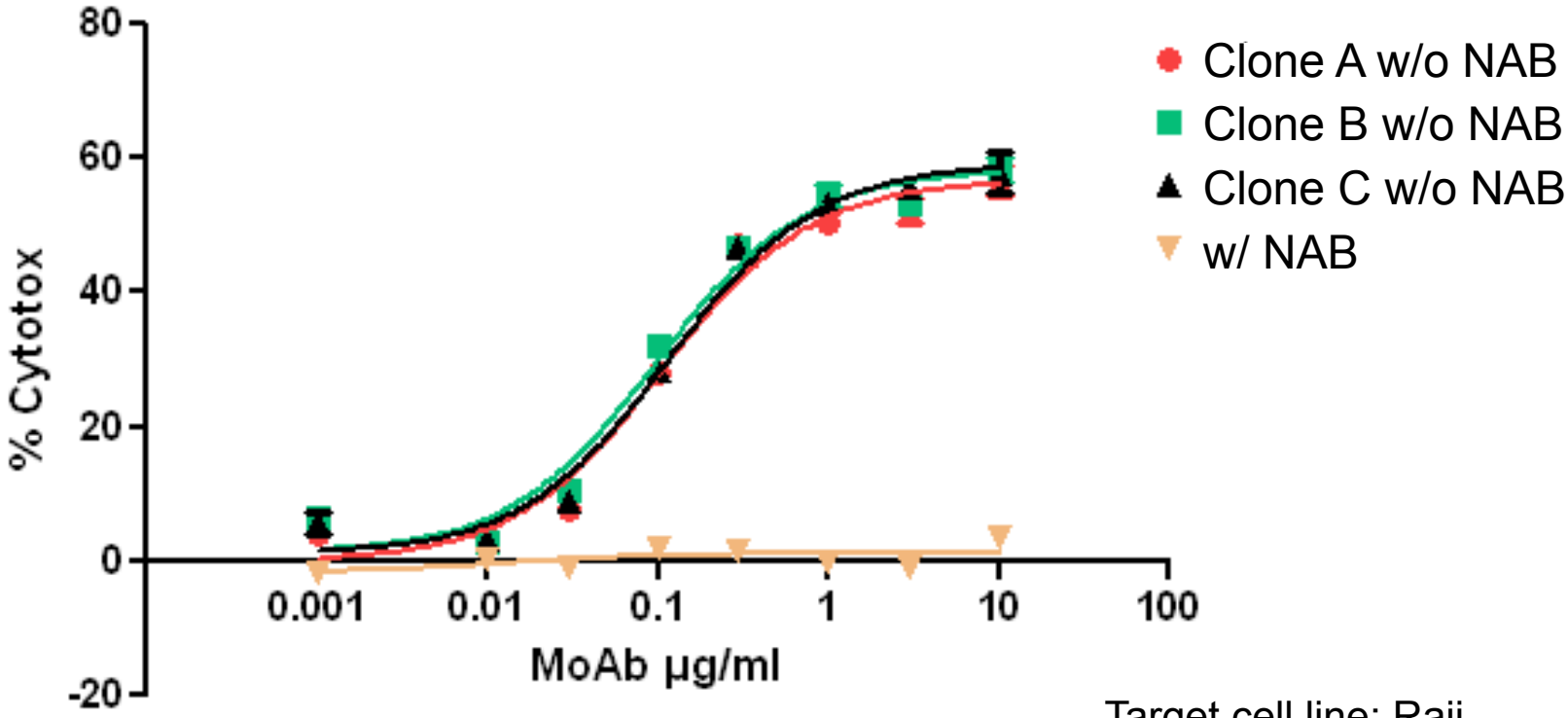
# Example: mab

# Inhibition of ADCC by anti-IS



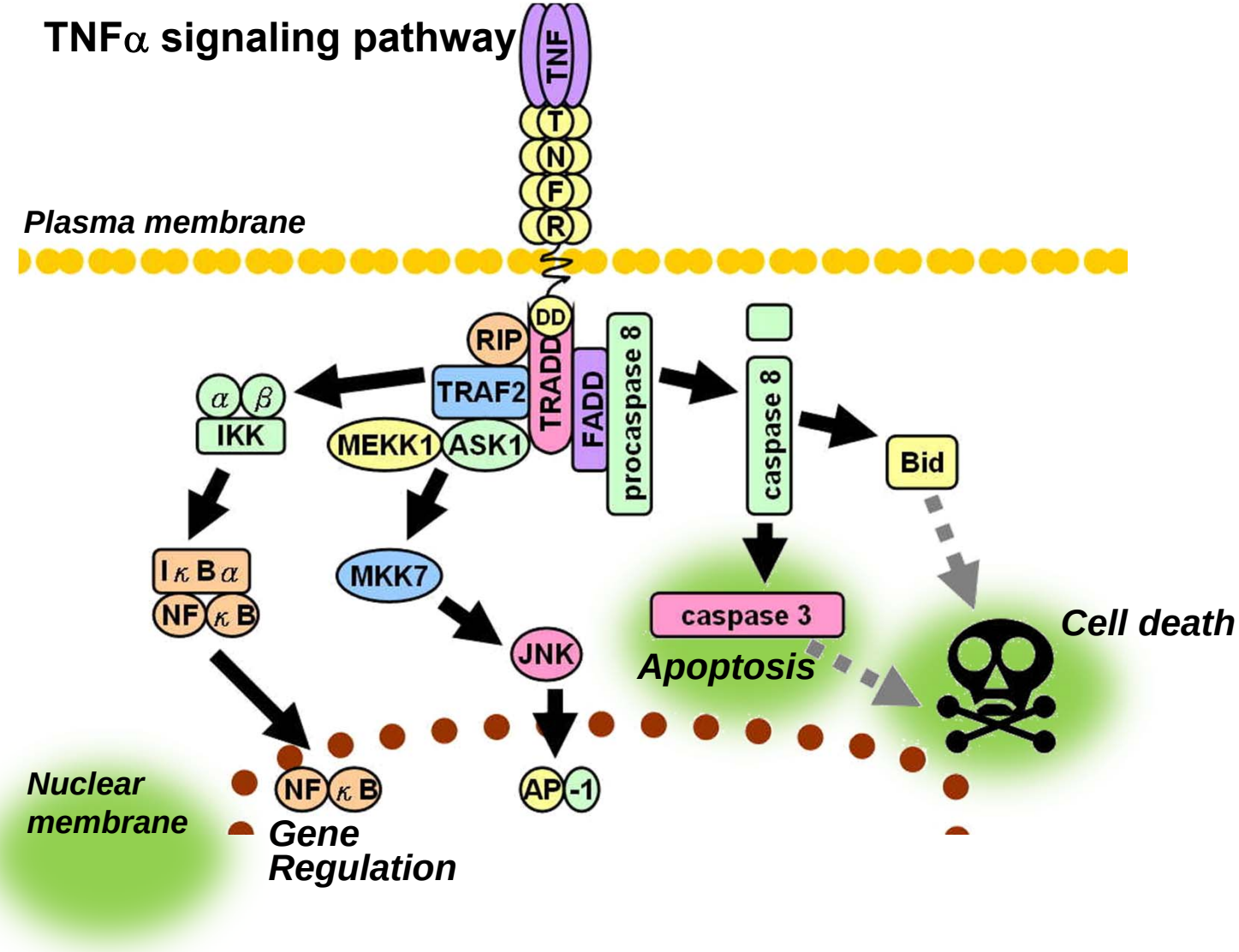

Target cell line: SK-BR3i
MRD: 2 %
Effector cell: CD16-NK
Drug: anti-Her2/neu

# Inhibition of CDC by anti-IS




Target cell line: Raji
MRD: 2 %
Effector: complement (10:1)
Drug: anti-CD20

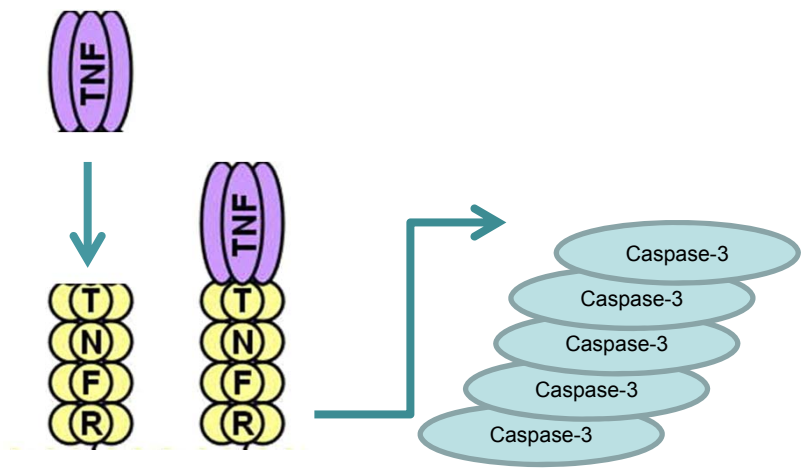
# Mechanism of action via TNFα signaling

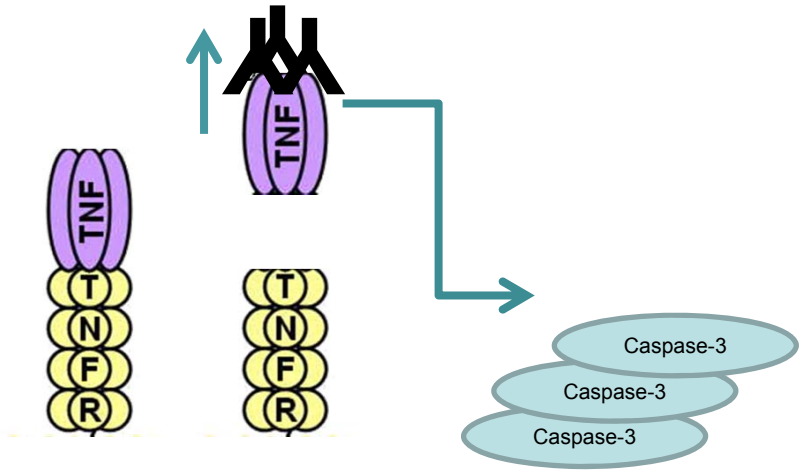


# TNFα blocker cell-based bioassay based on caspase 3 activity

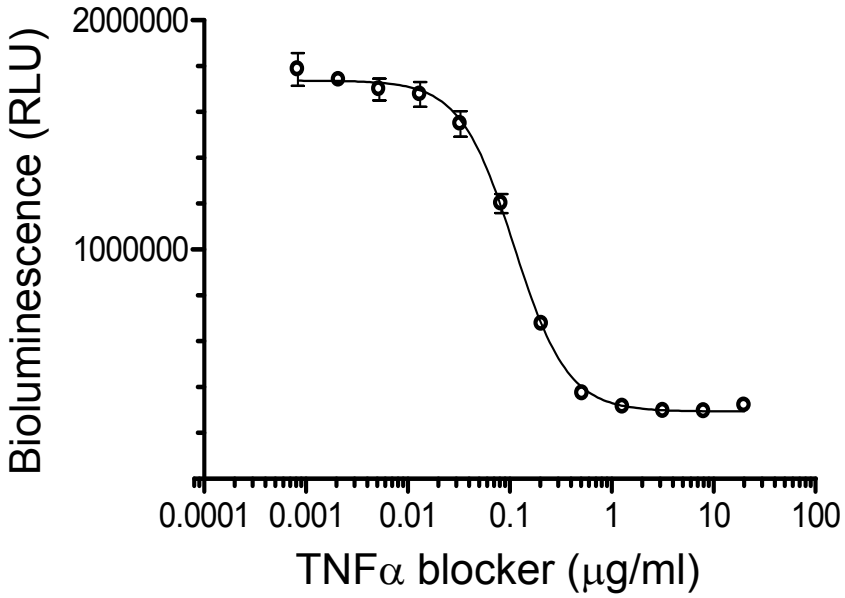
TNFα / TNFα receptor signaling via the apoptosis pathway increases caspase 3 activity

A TNFα blocker drug dose-responsively lowers caspase 3 activity of TNFα by blocking TNFα binding to receptors



# Bioluminescent caspase-based bioassay of TNFα blocker drug activity on TNFα signaling

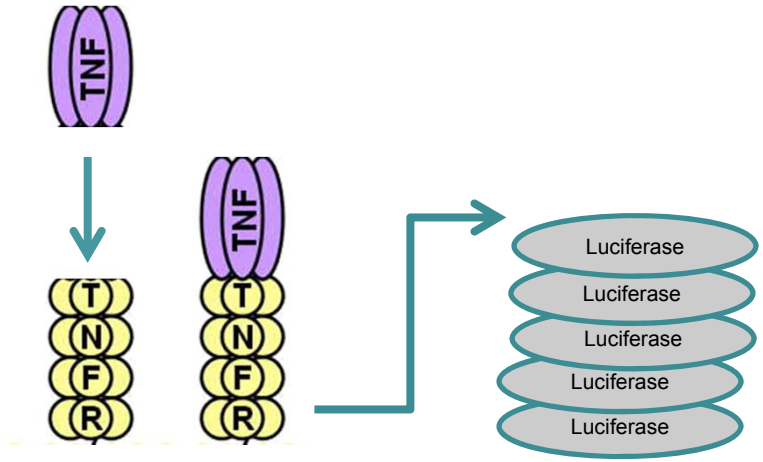


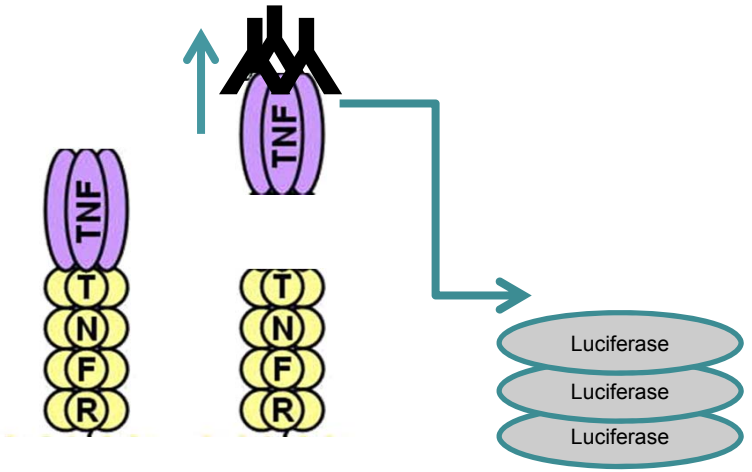

- Rapidly responsive human U937 cells in provide bioassay high consistency
- Bioluminescence readout provides excellent bioassay sensitivity and dynamic range
- Fast assay (2.5 hr response)

# TNFα blocker cell-based bioassay based on NF-κB luc reporter activity

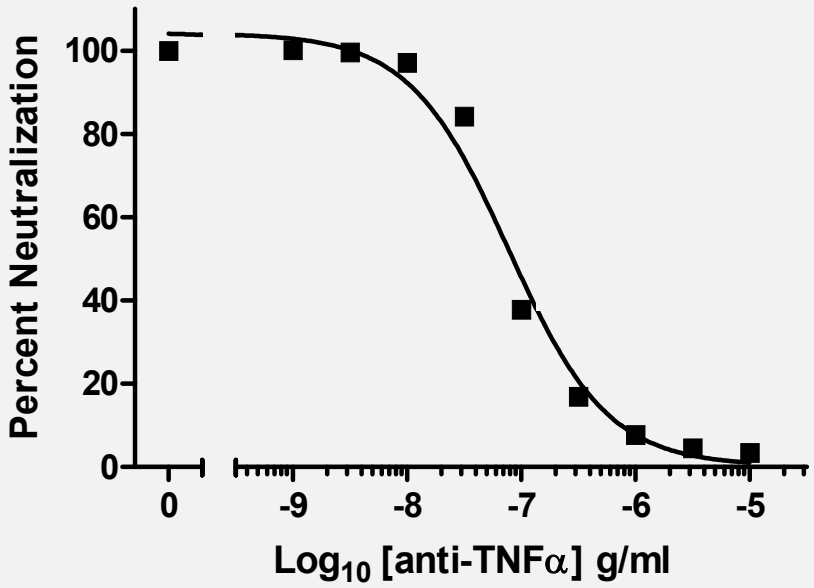
TNFα / TNFα receptor signaling via the NF-κB pathway increases gene expression driven by the NF-κB response element.

A TNFα blocker drug dose-responsively lowers NF-κB driven luciferase activity of TNFα by blocking TNFα binding to receptors



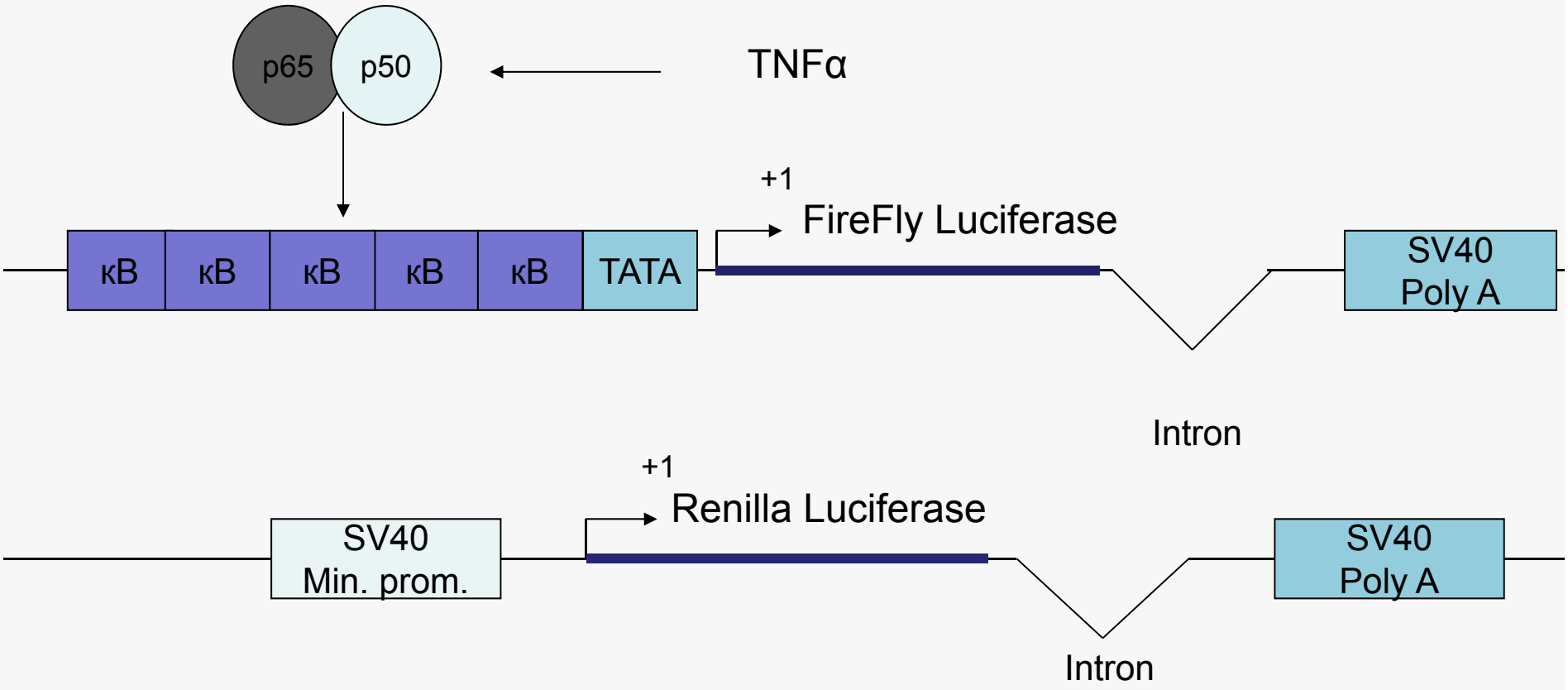



# Bioluminescent NF-κB reporter gene bioassay of TNFα blocker drug activity on TNFα signaling



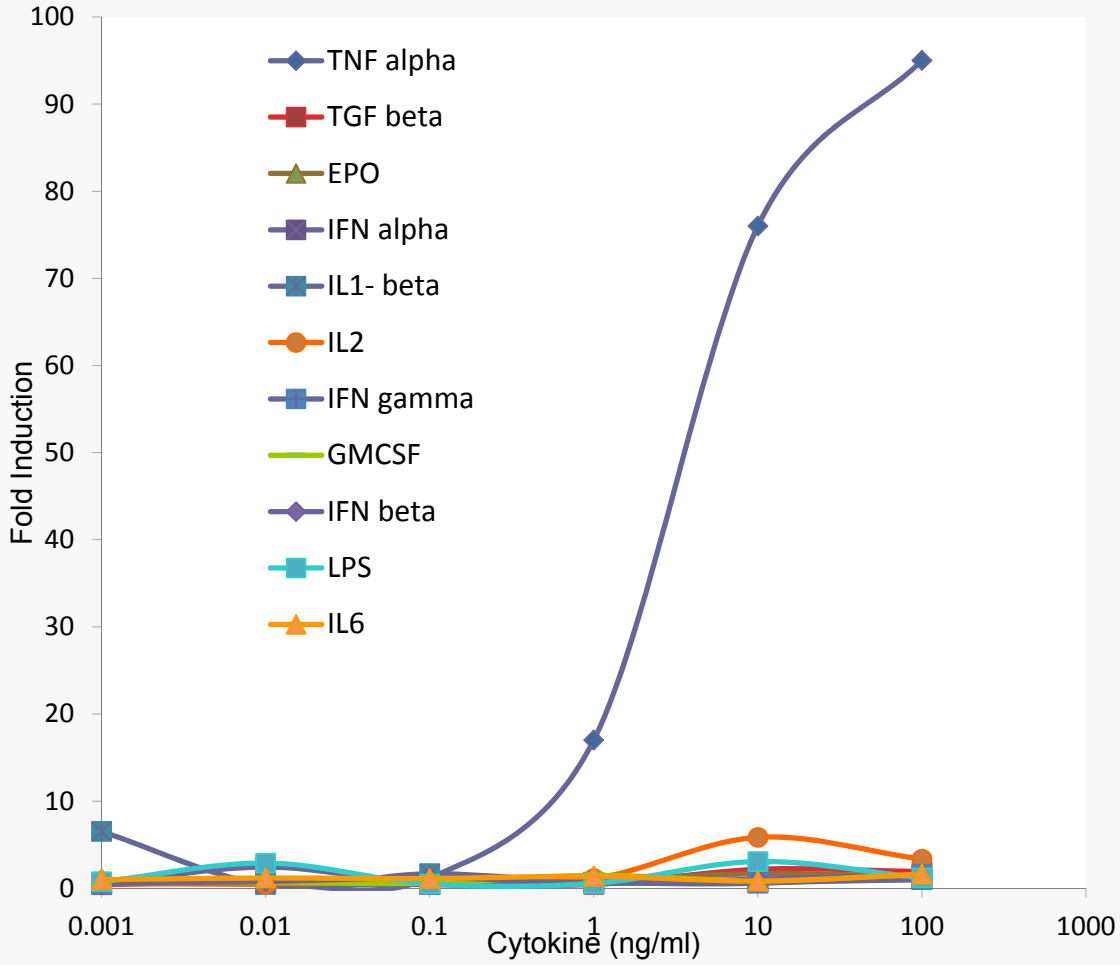

- Stably transfected human NF–κB HEK-293 cells provide high consistency
- Bioluminescence readout provides excellent bioassay sensitivity and dynamic range
- Fast assay (4 hr induction of NF-κB driven luciferase expression)

# TNFα Resp Reporter Gene Construct



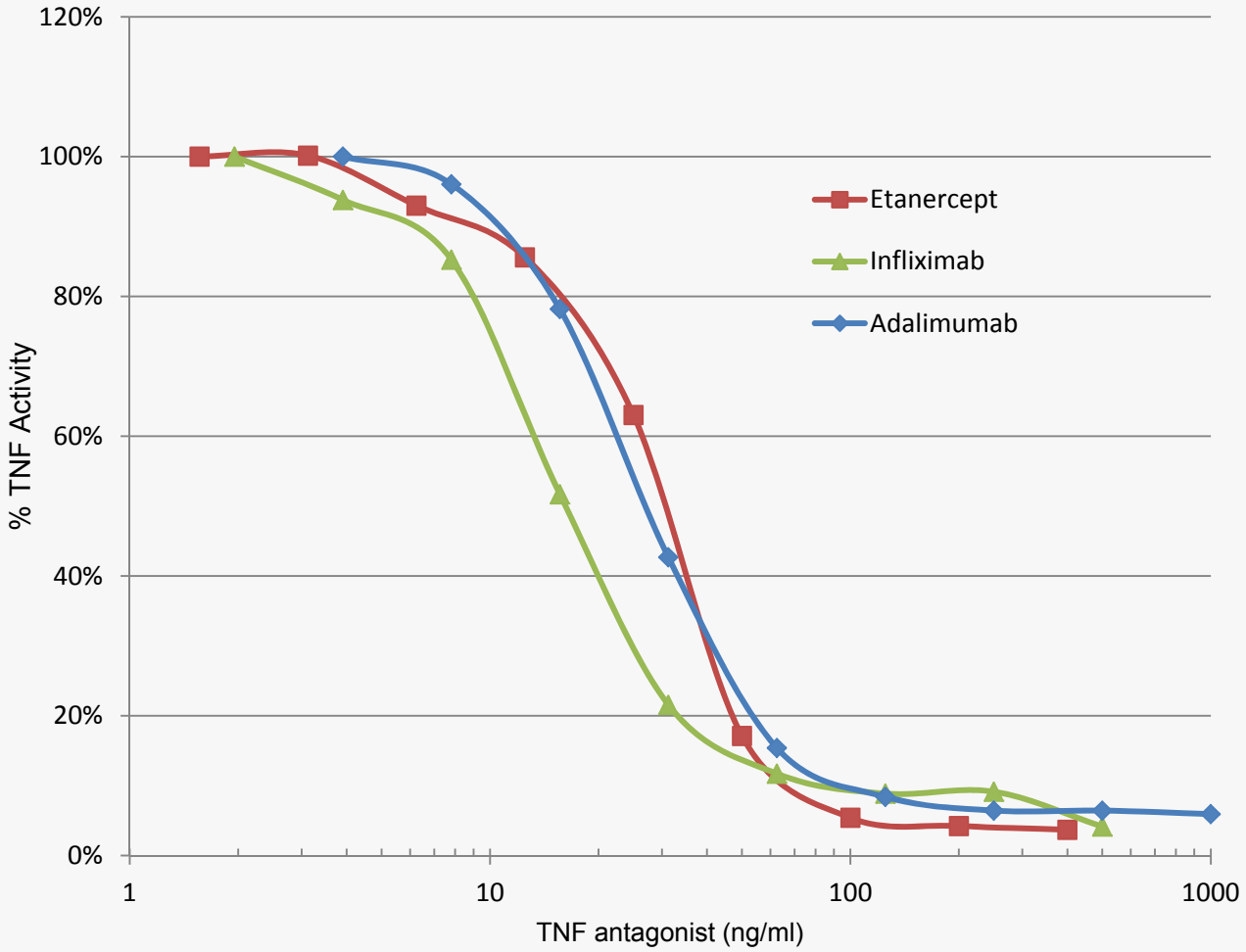

Lallemand C, et al, **Tovey** MG. J Immunol Methods. 2011;373:229-39

# Specificity of TNFα-assay



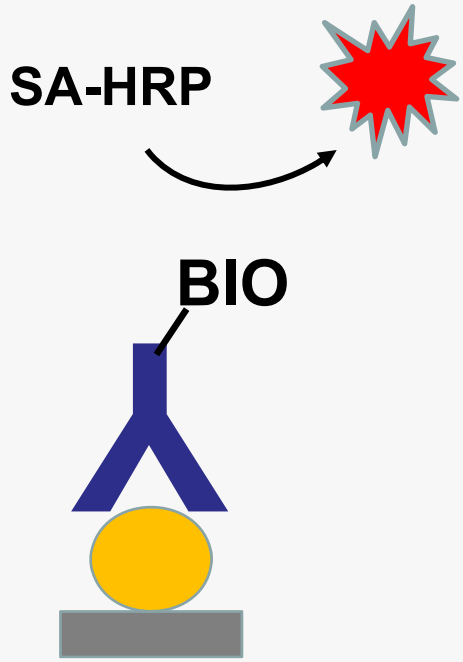

Lallemand C, et al, **Tovey** MG. J Immunol Methods. 2011;373:229-39

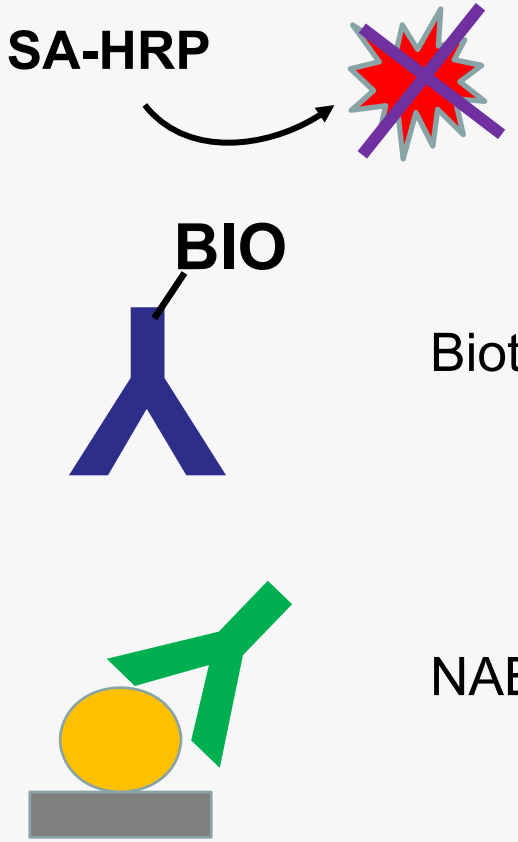
# Anti-TNFα-NAB analysis




Lallemand C, et al, **Tovey** MG. J Immunol Methods. 2011;373:229-39

# Competitive ligand binding assays




Biotinylated drug

NAB against drug

# Validation data of CLB

| | Run1 | | Run 2 | | Run 3 | | Run 4 | | Mean | |
|---|---|---|---|---|---|---|---|---|---|---|
| **NAB** | **Signal** | **%CV** | **Signal** | **%CV** | **Signal** | **%CV** | **Signal** | **%CV** | **Signal** | **%CV** |
| **125** | 0.476 | 4.20 | 0.802 | 6.10 | 0.951 | 3.1 | 0.687 | 13.6 | **0.729** | 0.2 |
| **31** | 0.923 | 2.20 | 1.056 | 0.40 | 1.353 | 2.8 | 1.141 | 6.6 | **1.118** | 0.2 |
| **16** | 0.980 | 2.00 | 1.066 | 0.60 | 1.382 | 2.7 | 1.215 | 3.1 | **1.161** | 0.2 |
| **8** | 1.029 | 1.00 | 1.121 | 0.70 | 1.376 | 4.0 | 1.228 | 3.9 | **1.189** | 0.1 |
| **2** | 1.069 | 0.90 | 1.194 | 0.00 | 1.485 | 1.0 | 1.266 | 3.8 | **1.254** | 0.2 |
| **0** | 1.051 | 1.90 | 1.154 | 3.90 | 1.512 | 1,1 | 1.304 | 2.5 | **1.255** | 0.2 |
| **NC** | 1.142 | 2.60 | 1.212 | 1.80 | 1.476 | 2,9 | 1.134 | 1.2 | **1.241** | 0.1 |
| **blank** | 0.012 | - | 0.015 | - | 0.016 | 9.4 | 0.017 | - | **0.015** | - |
| **bio drug** | 1.136 | 2.60 | 1.713 | 1.80 | 1.823 | 2.7 | 1.615 | 3.4 | **1.572** | 0.3 |

# Conclusion

- Assays for the detection of neutralizing antibodies should be included in the cascade of immunogenicity assessment.
- Neutralizing antibodies (NAB) are generally of more concern than binding antibodies (BAB).
- The detection of NAB can be performed by cell-based assays (CBA) or by non-cell-based competitive ligand binding assays (CLBA).
- FDA prefers CBA because these more realistically reflect the in vivo situation.
- Sometime cell-based assays are more difficult and tedious to establish. Recombinant cell lines / reporter gene readouts may be an alternative for the NAB analysis if other cell-based assay are not available.

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