Establishment of a Cell-Based Assay Specific for a Growth Factor that Shares a Common Receptor Chain and Overlapping Biological Activities with Other Cytokines and Growth Factors

Michael G Tovey,

INSERM Director of Research, Laboratory of Biotechnology & Applied Pharmacology,
ENS CACHAN

tovey@vjf.cnrs.fr

Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF)

- GM-CSF is a hematopoietic growth factor that plays a central role in in the generation of neutrophils, macrophages, and DCs
- GM-CSF acts together with IL-3 and IL-5 to regulate the survival proliferation, differentiation, and functional activation of hematopoietic cells
- GM-CSF also regulates its own activity by via the induction of CIS, a SOCS family member SH2-domain protein that inhibits Jak2/STAT5 phosphorylation & signaling.
- CIS and SOCS3 also regulate EPO induced Jak2/STAT5 signaling

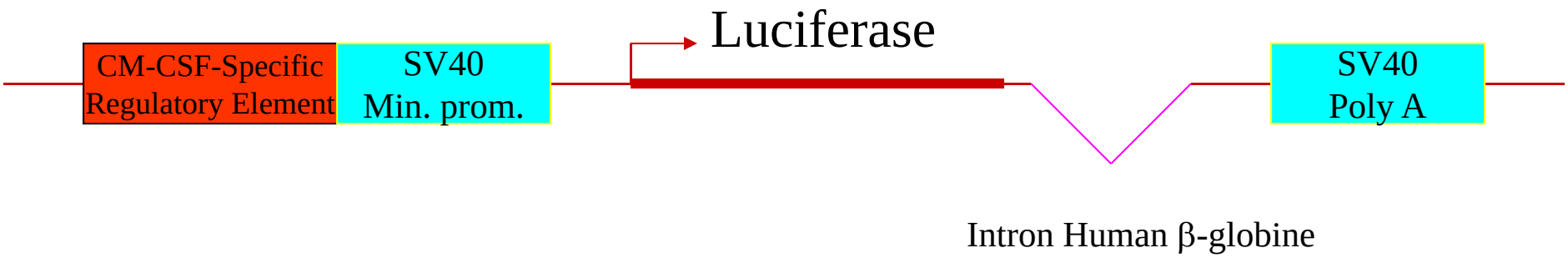
Quantification of GM-CSF Activity

- Current methods for quantifying human GM-CSF activity, are bioassays based on the ability of GM-CSF to support the proliferation of cell lines such as TF-1, or UT-7, that require GM-CSF for their growth
- Due to overlapping biological activities, IL-3 or EPO, can also support the proliferation of these cells and act synergistically together with GM-CSF.
- M-CSF & IL-1 can also enhance GM-CSF dependent cell proliferation
- TGF-β and IFNα, or IFN β, can antagonize this activity
- IL-3 & IL-5 share a common receptor βc chain with GM-CSF
- Thus, proliferation based assays for GM-CSF are subject to non specific interference.

Development of a Cell-based Assay Specific for GM-CSF: *Strategy*

- Use a cell line that possesses functional GM-CSF receptors, but does *not* require GM-CSF or other related growth factors for proliferation
- U937 cells possess functional GM-CSF receptors but not express functional EPO receptors
- U937 cells do not require GM-CSF, EPO or other related growth factors for proliferation
- Transfect U937 cells with a GM-CSF responsive reporter-gene construct

Reporter-Gene Assay: Construction



However,

GM-CSF, IL-3, & IL-5 share a common receptor βc chain

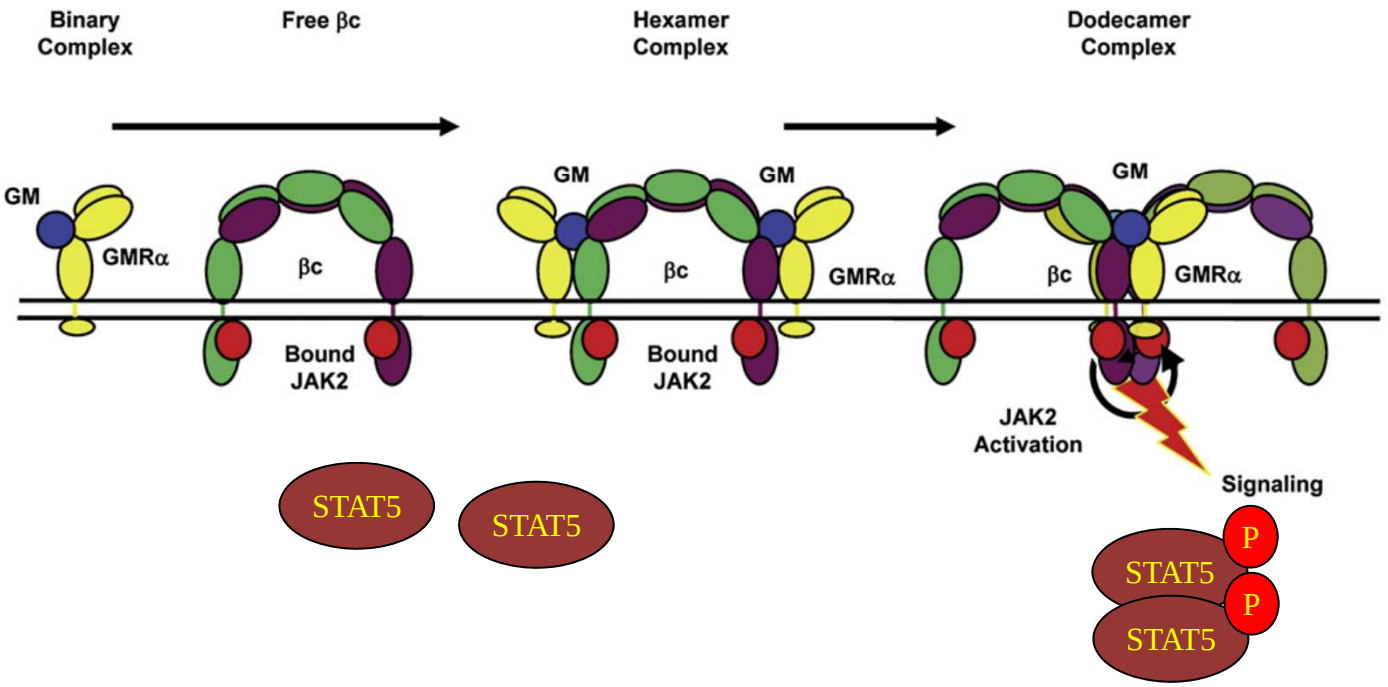
GM-CSF Receptor

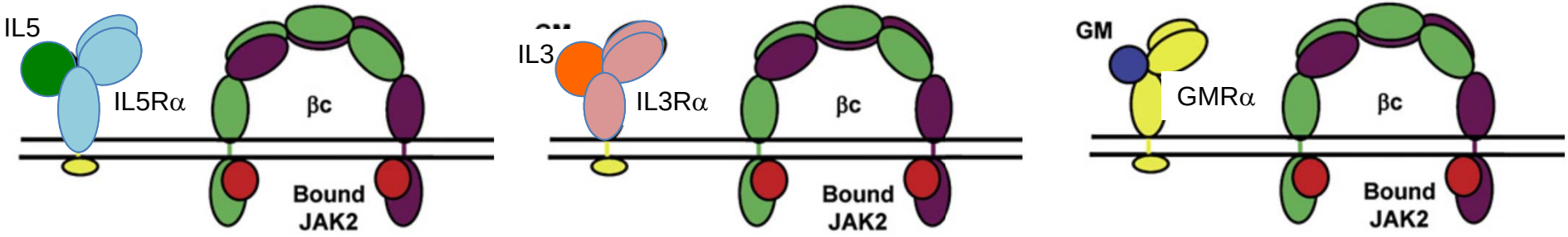
- GM-CSF binds to a heterodimeric receptor comprised of a GM-CSF specific α subunit and a common receptor βc chain that is shared with IL-3 & IL-5
- GM-CSF receptor does not possess intrinsic tyrosine kinase activity; associates with Jak2 required for βc trans-phosphorylation, initiation of signaling, & biological activity
- Receptors for GM-CSF, IL-3, & IL-5 are expressed at very low levels (100-1,000 receptors/cell)
- GM-CSF, IL-3, & IL-5 each bind with low affinity to their specific Rα chain (Kd = 0.2 – 100 nM)
- In the presence of the βc receptor chain each cytokine binds with high affinity (Kd = 100 pM) resulting in dimerization of both sub-units and receptor activation

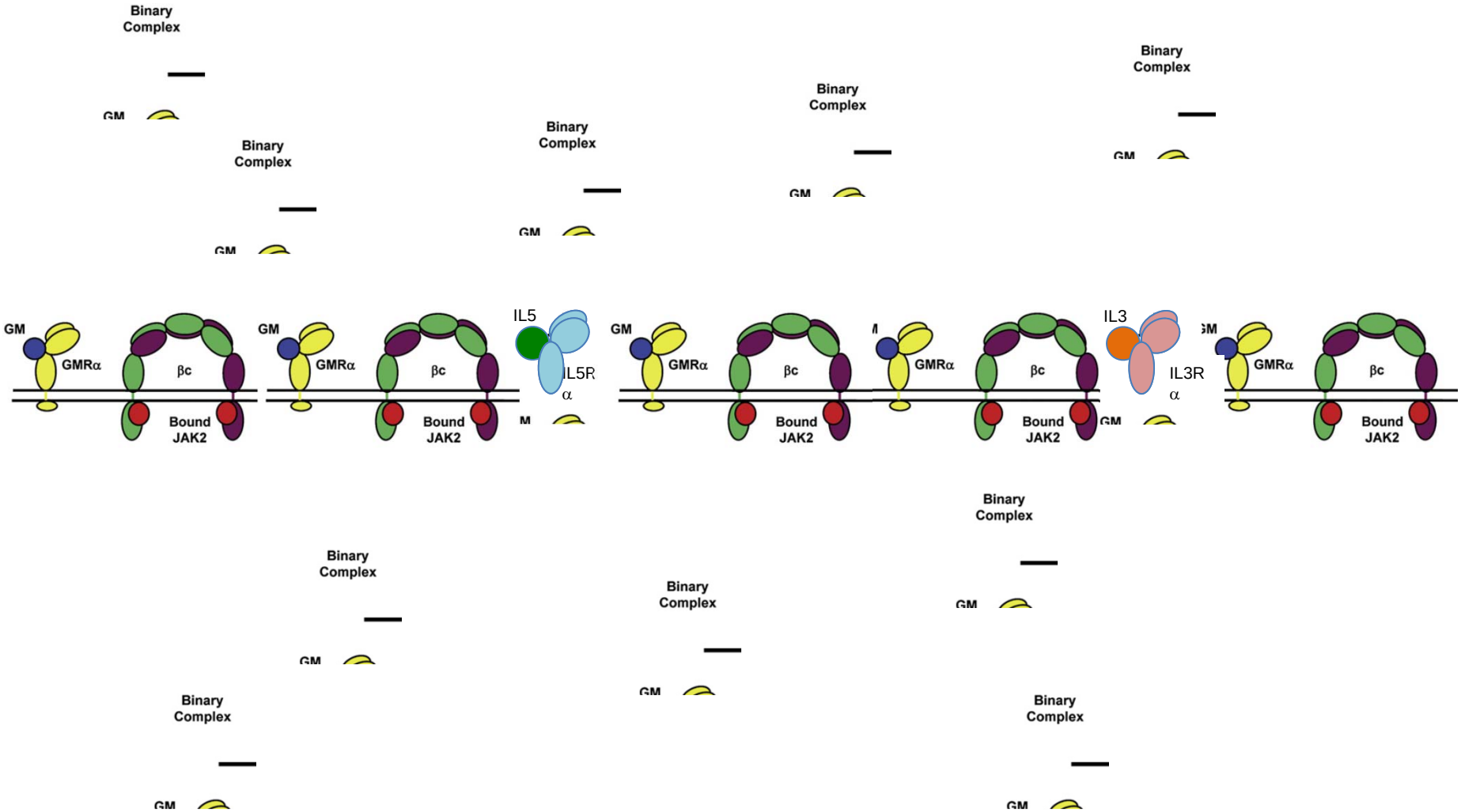
Development of a Cell-based Assay Specific for GM-CSF: *Strategy*

- Overexpress the GMRα, GM-CSF ligand-specific binding sub-unit, of the human GM-CSF receptor
- *Hypothesis*, GMRα receptor chain will compete with IL-3 or IL-5 specific binding sub-units for the pool of the βc signaling receptor sub-unit common to the GM-CSF, IL-3, and IL-5 heterodimeric receptors.

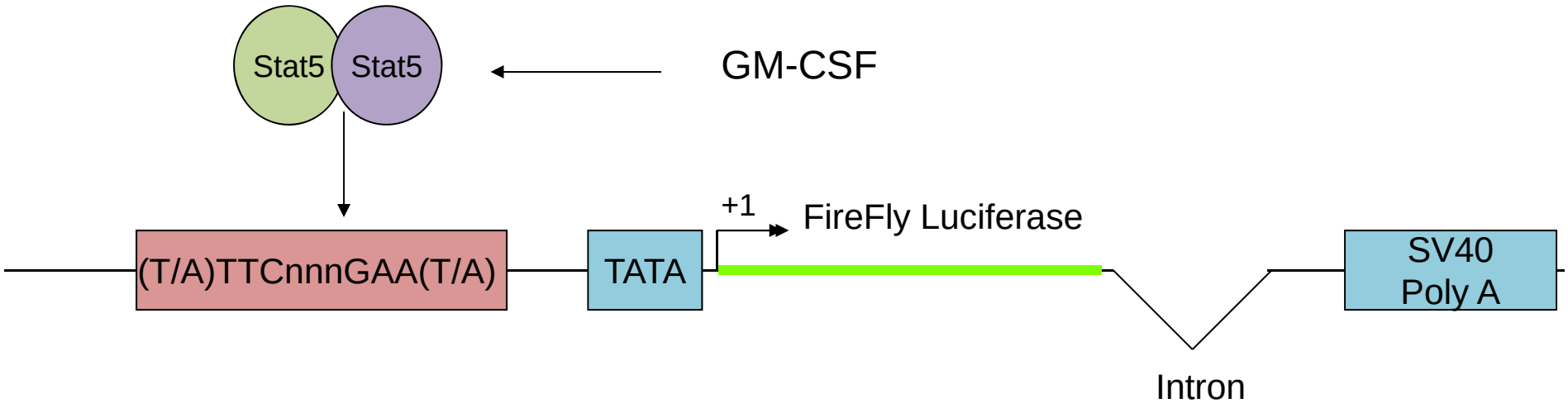
GM-CSF Receptor Structure and Signal Transduction




IL5
IL5Rα
βc
Bound
JAK2
IL3
IL3Rα
βc
Bound
JAK2
GM
GMRα
βc
Bound
JAK2


Binary Complex
GM
GMRα
βc
Bound JAK2
IL5
IL5R α
IL3
IL3R α

GM-CSF Gene Reporter Assay (FireFly Luciferase)



STAT5 Consensus Sequence:

STAT5 con. Ax4

TTCCCCGAAATGATGAGCTAGGAGCCTGATTTCCCCGAAATGATGAGCTAGGAGCCTGATTTCCCCGAAATGATGCTAGGAGCCTGATTTCCCCGAAATGATctGtTAG

STAT5 con. Bx6

GAGGCTCTGATTTCCGGGAAACTGATTCCCGGAATACGTTTTCCGGGAATACGTTTCCGGGAAACGTATTCCGGGAAAACTGATTCCGGGAAATGATCTGTTAG

STAT5 con. Cx6

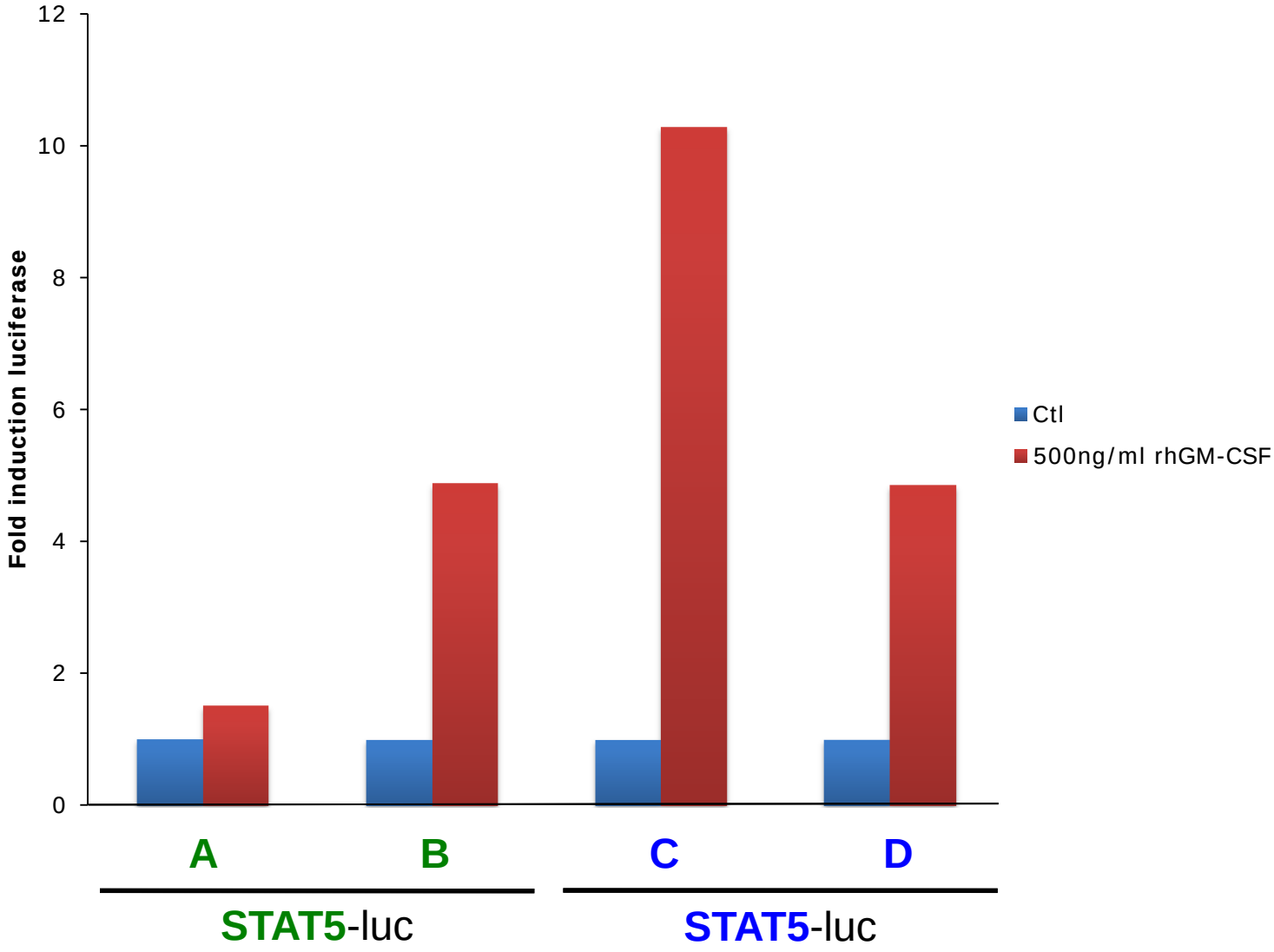
GATTTCTAGGAATTCttctcagaaGAATTCttctgagaaGAATTCttctcagaaGAATTCttctgagaaGAATTCttctcagaaGGAATTCAAATCG

STAT5 con. Dx4

GATTTCTAGGAATTCAAATCGGATCTAGATTTCTAGGAATTCAAATCGGATCTAGATTTCTAGGAATTCAAATCGGATCTAGATTTCTAGGAATTCAAATCG

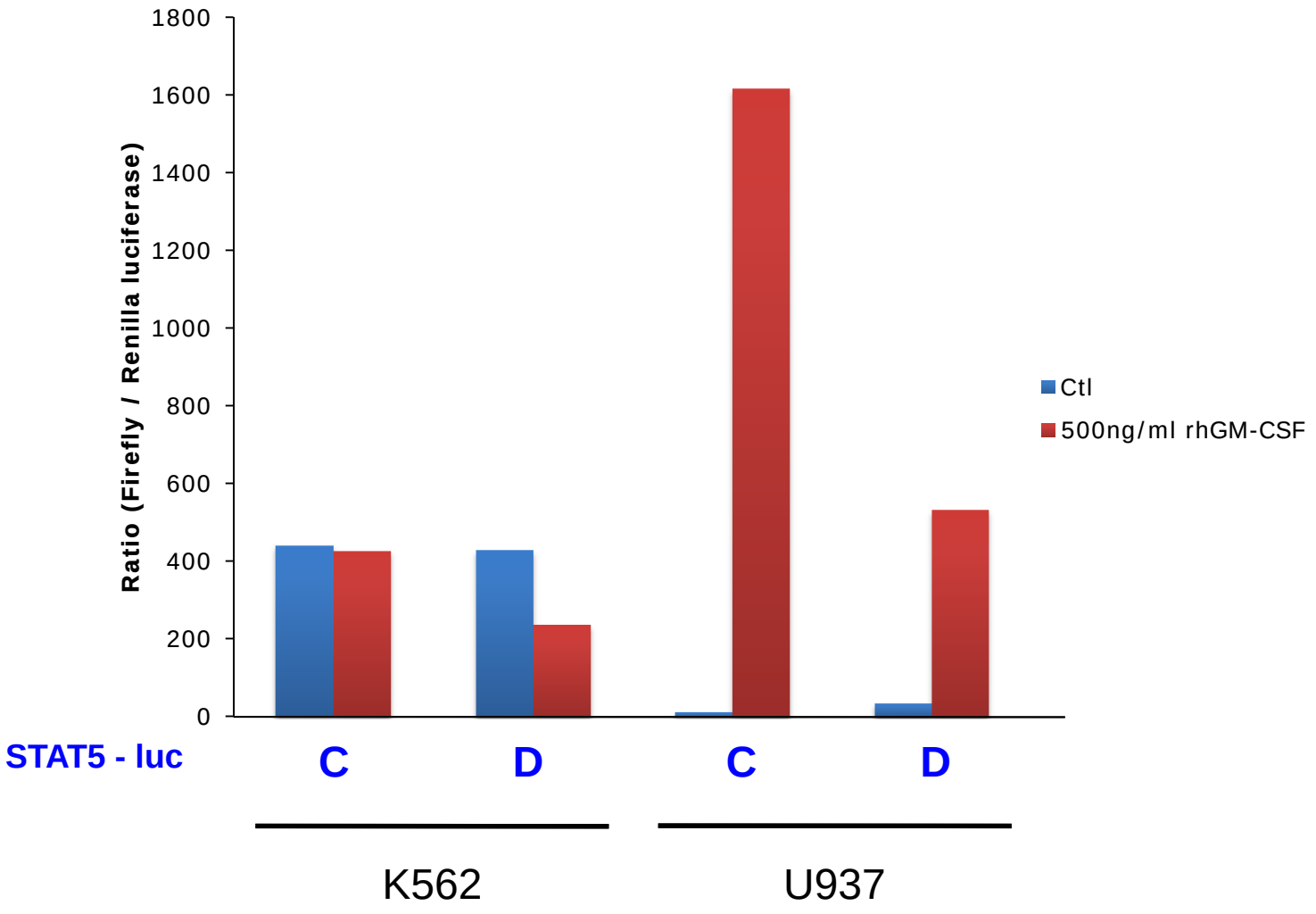
STAT 5 Reporter Gene Constructs – Firefly luciferase A, B, C or D

Transcient transfection in U937 cells



STAT5 Reporter Gene – Firefly luciferase (C or D) relative to Renilla luciferase expression

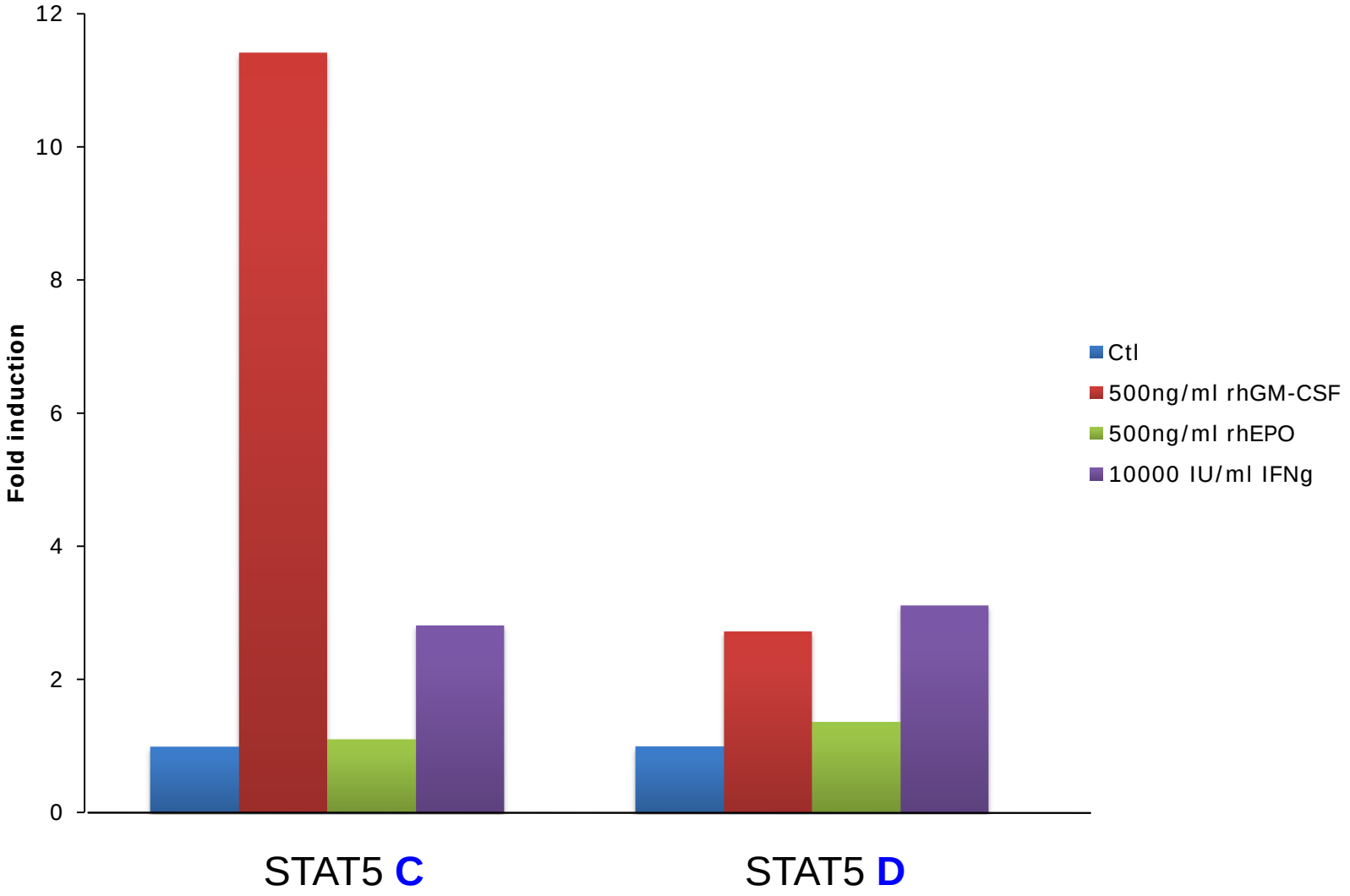
transient transfection in K562 and U937 cells



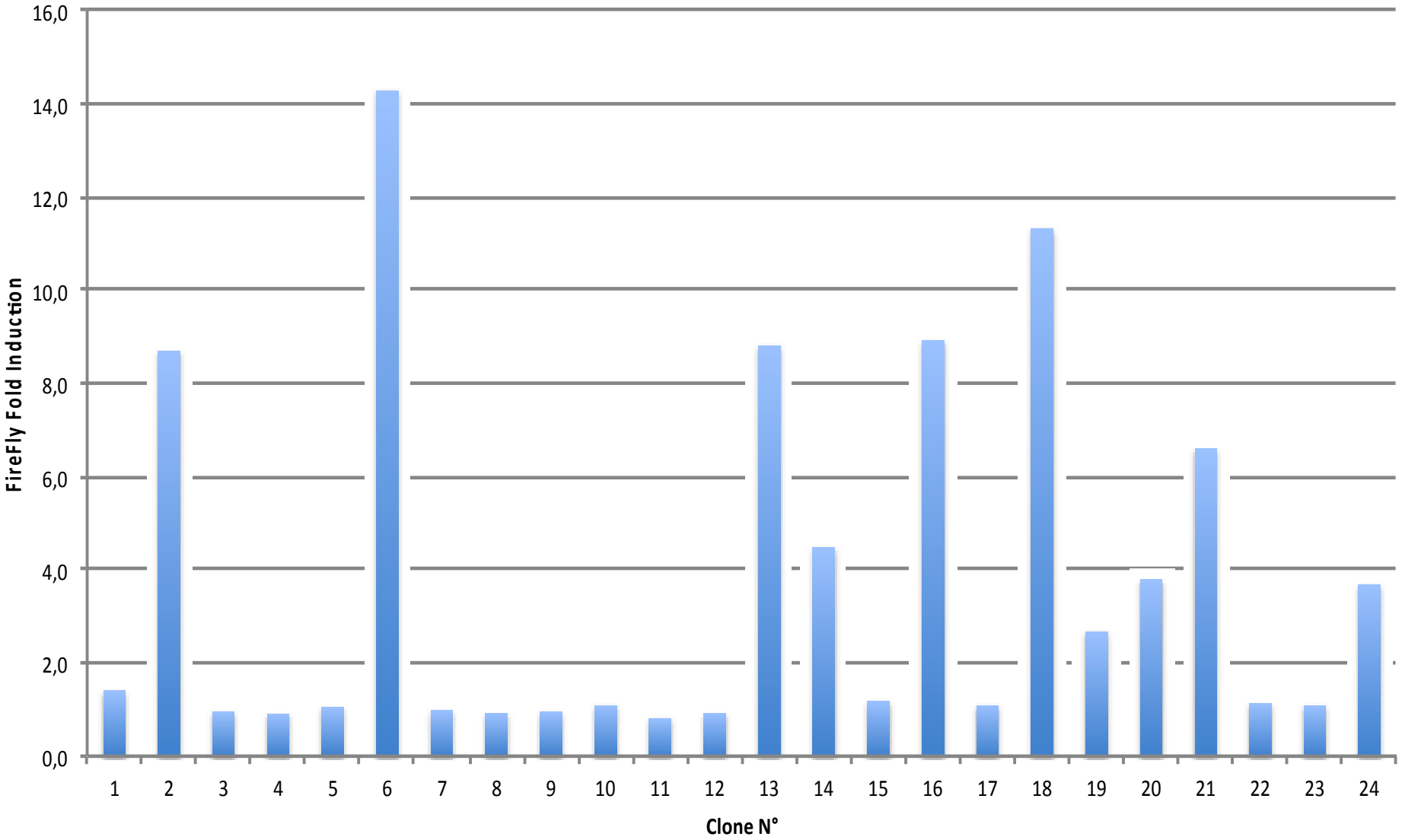
STAT5 is expressed constitutively in K562 cells

Effect of rhEPO and rhIFNγ on **STAT5** – luciferase (**C** or **D**) Reporter Gene

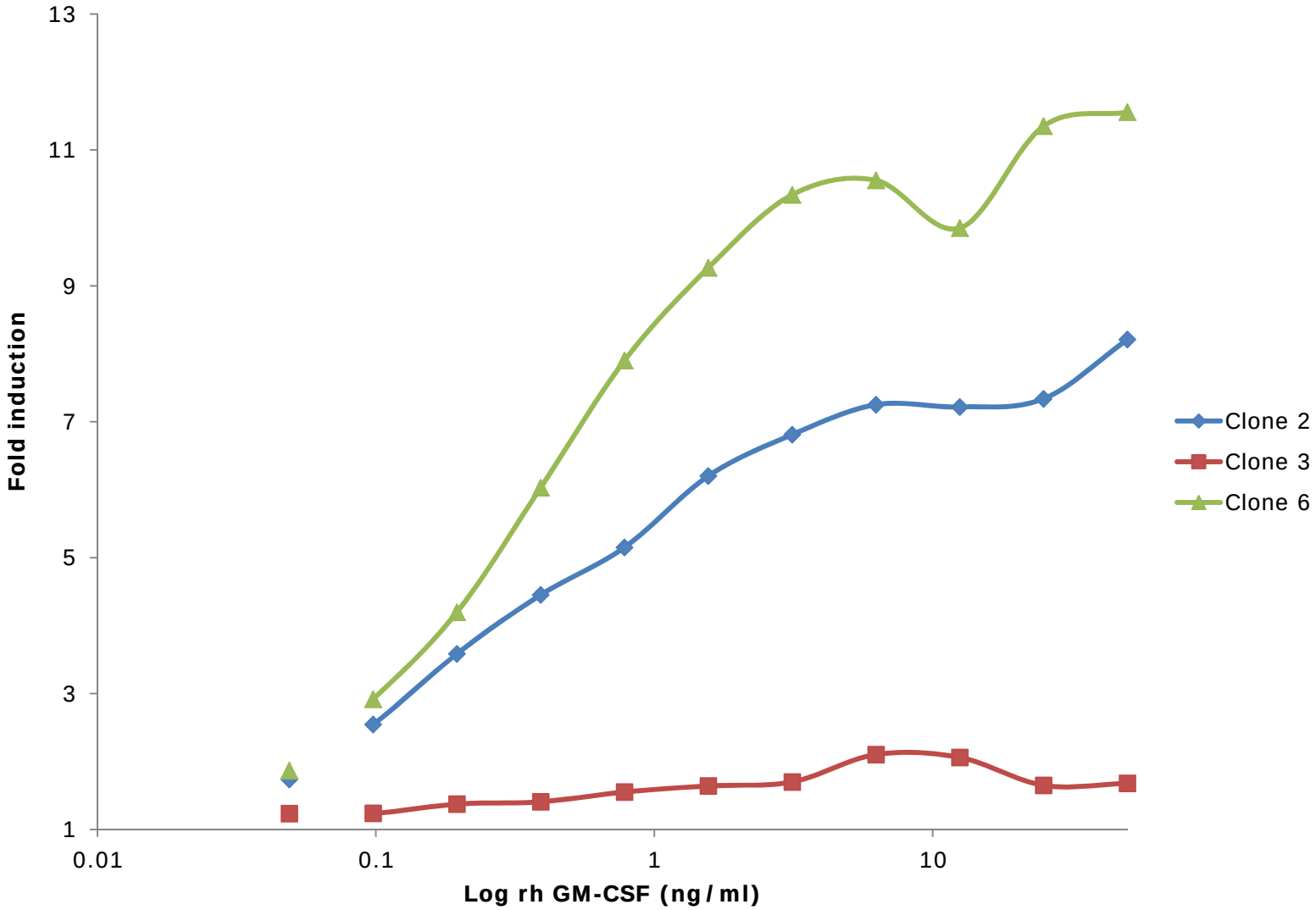
Transcient transfection in U937 cells



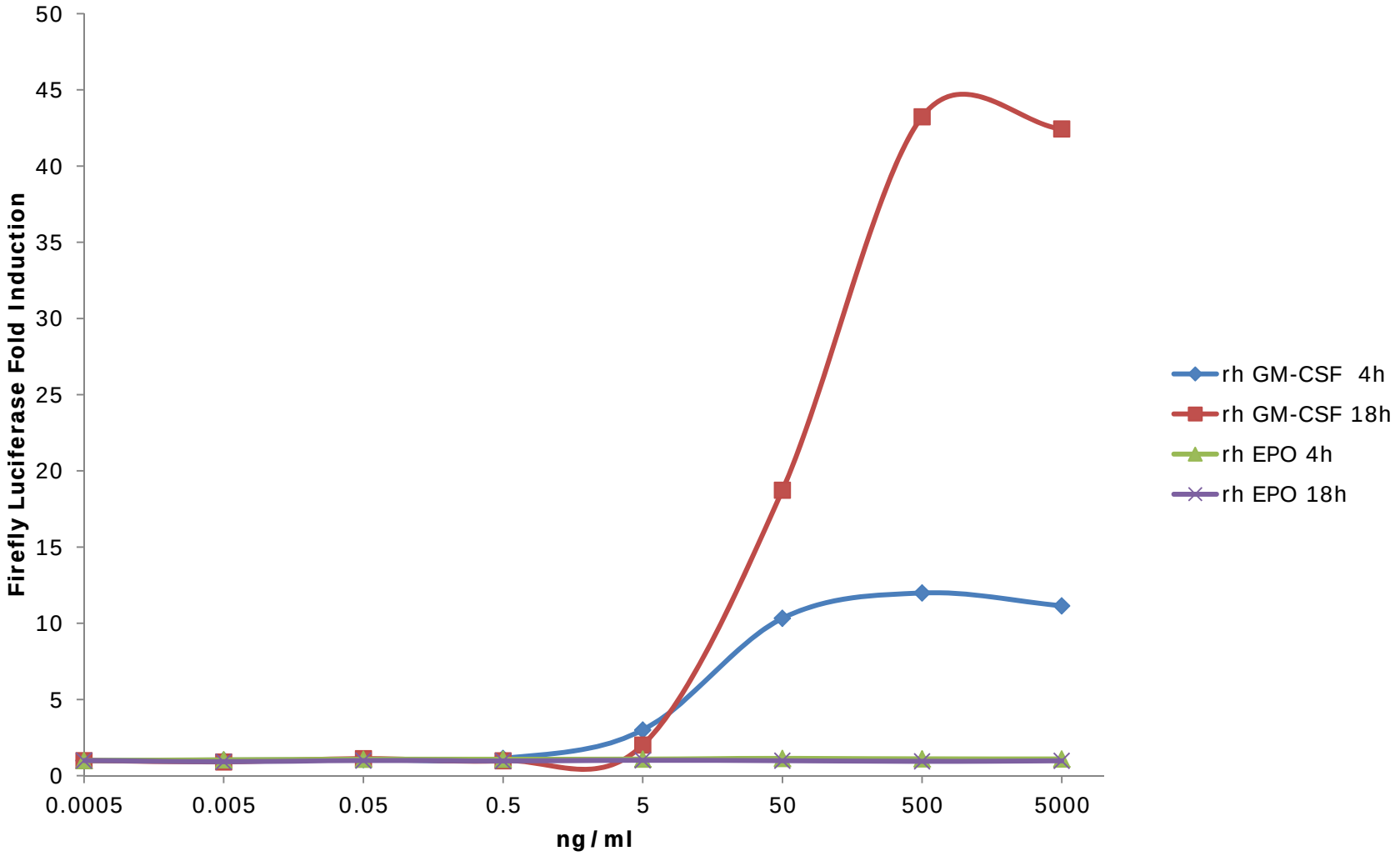
Analysis of Clones Transfected with the US5-Luc Construct



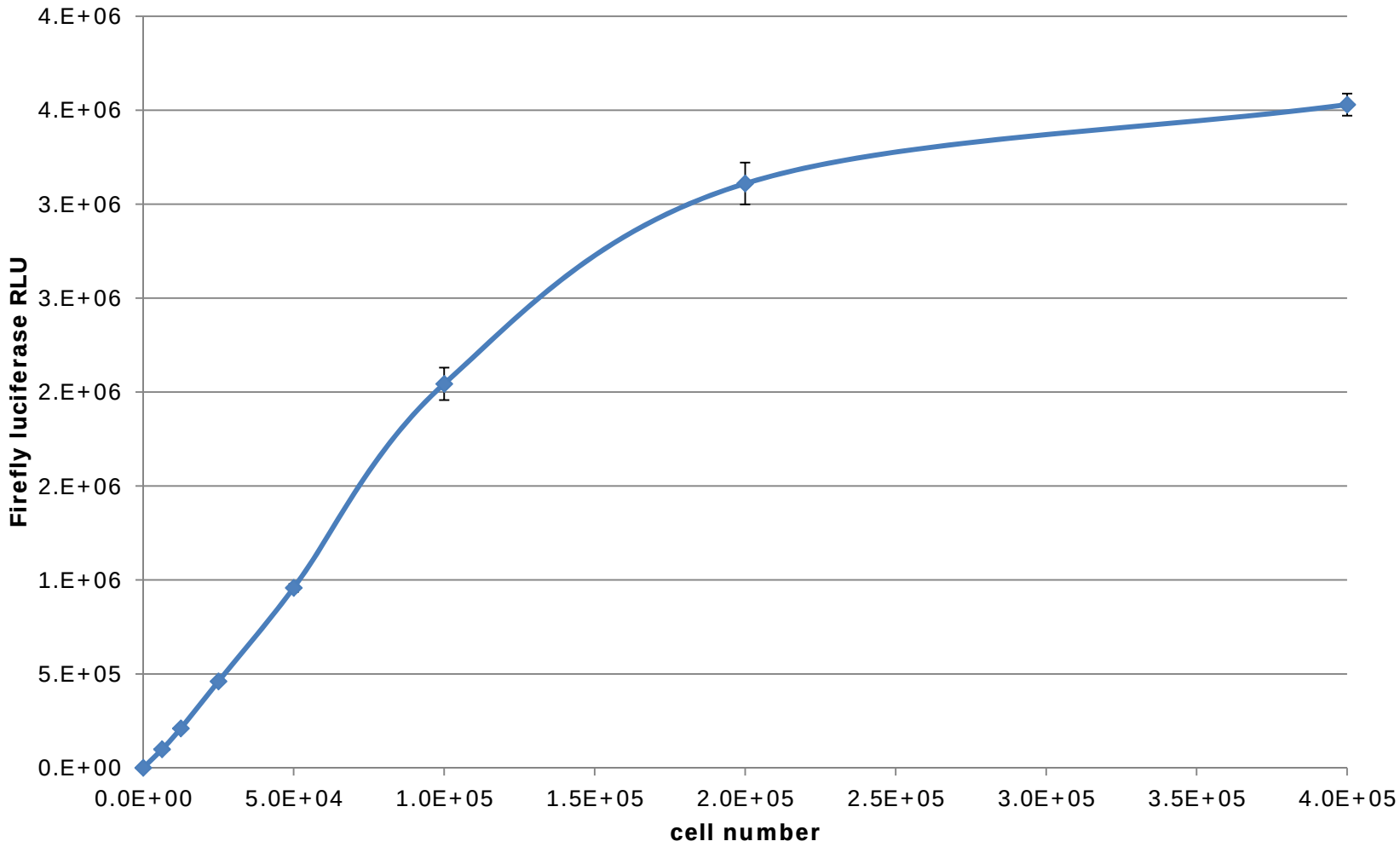
Selection of stable clones transfected by **STAT5**-luc **C** in U937 cell line

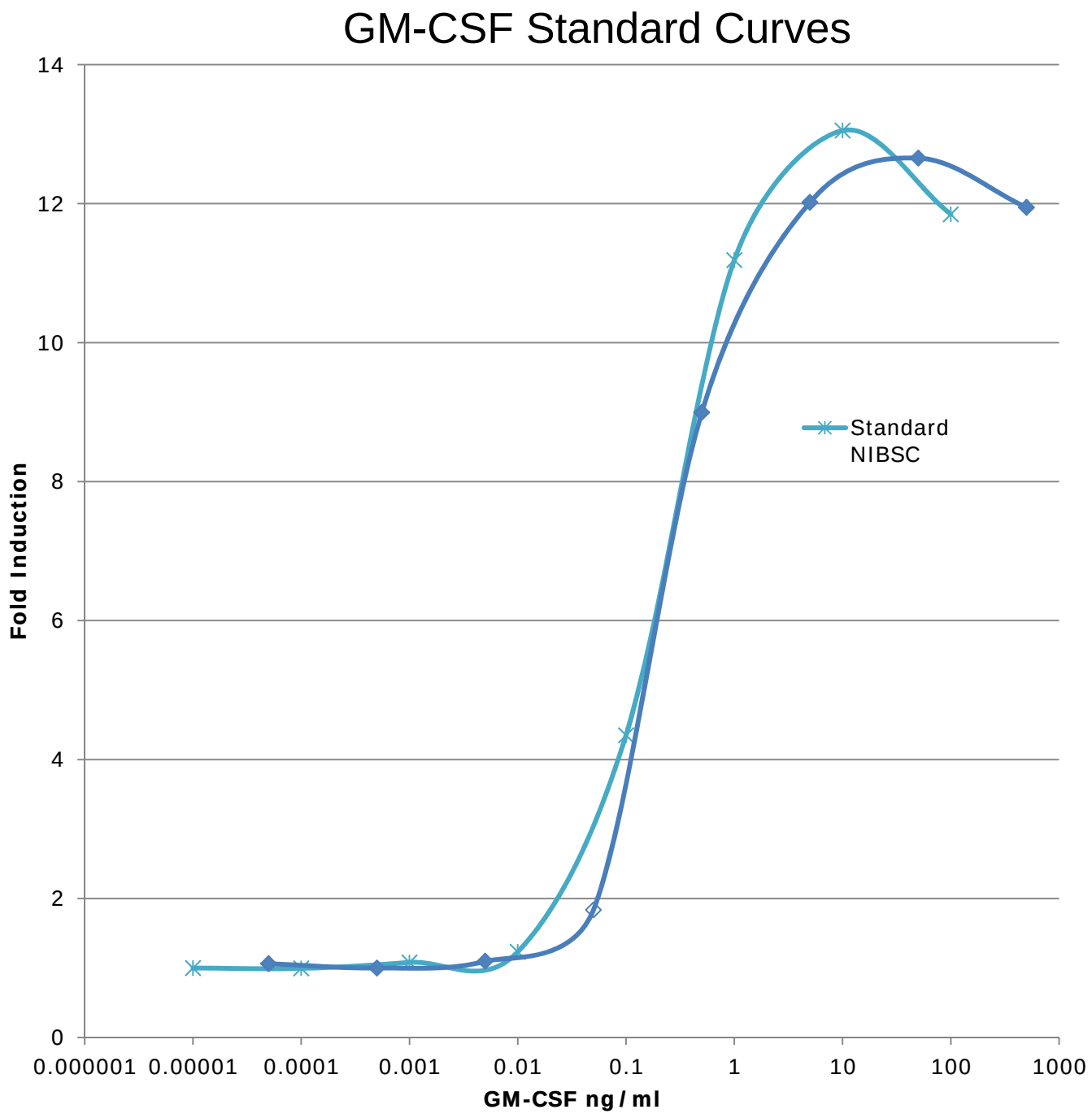


Firefly Luciferase induction in U937/STAT5-luc stable cell line

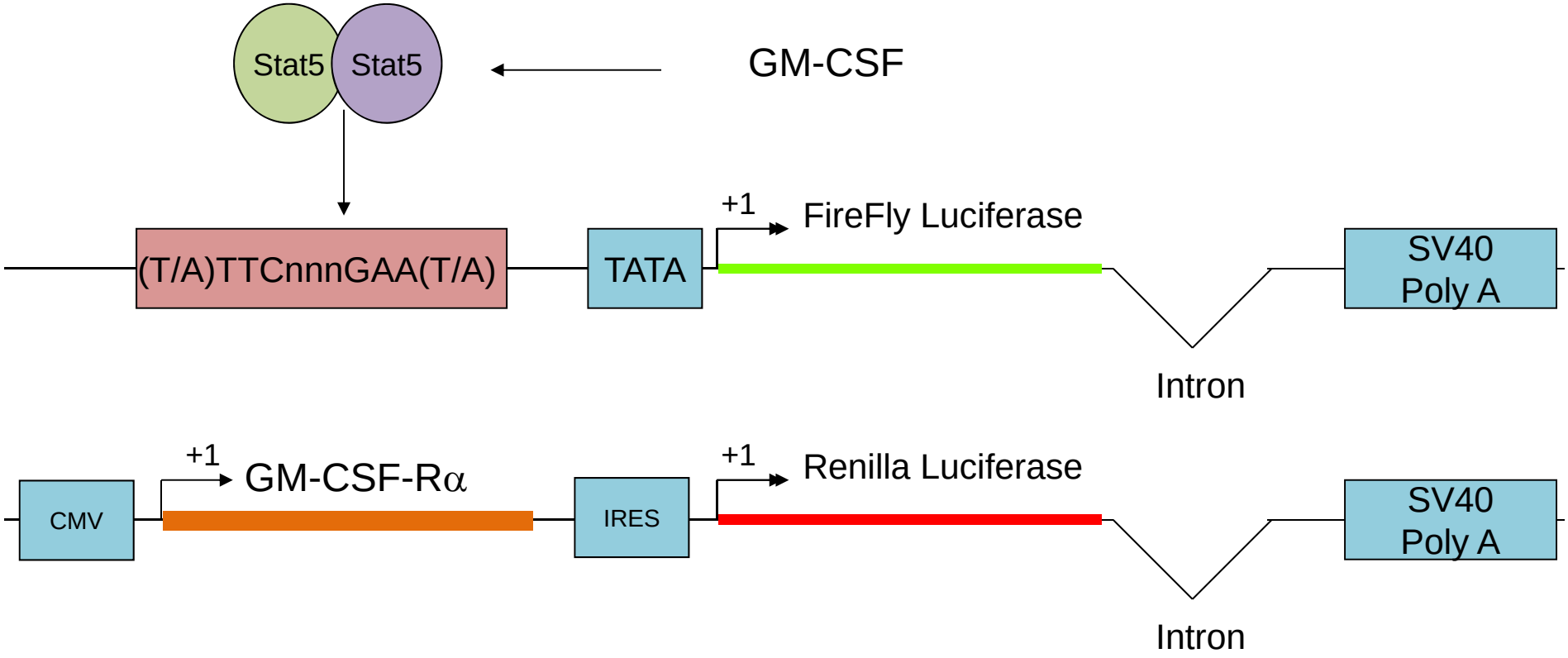


Cell number optimisation for the GM-CSF assay
Stimulation with 200ng/ml rhGM-CSF (Gibco®)

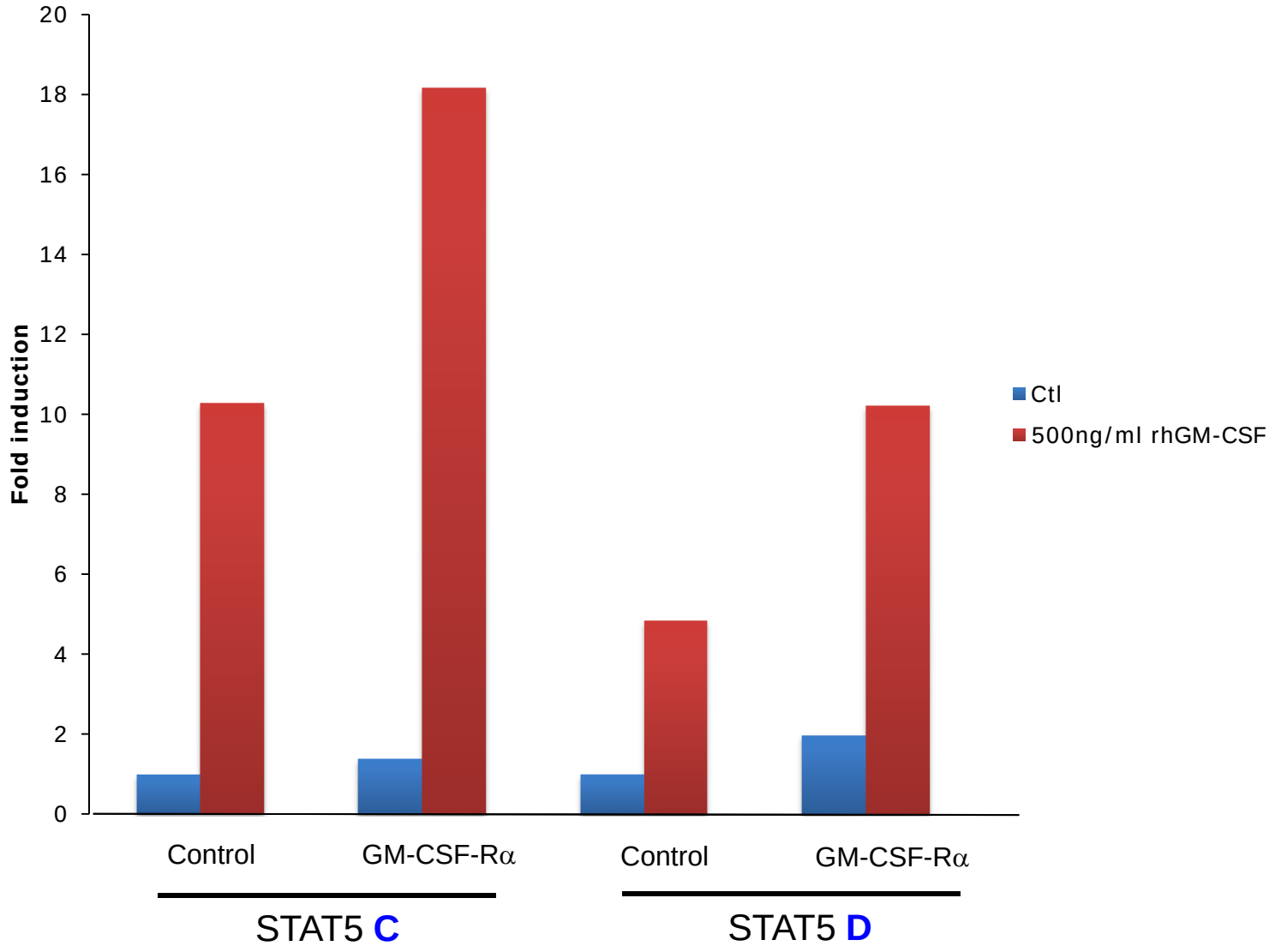



GM-CSF Standard Curves
14
12
10
8
6
4
2
0
Fold Induction
0.000001
0.00001
0.0001
0.001
0.01
0.1
1
10
100
1000
GM-CSF ng / ml
Standard
NIBSC

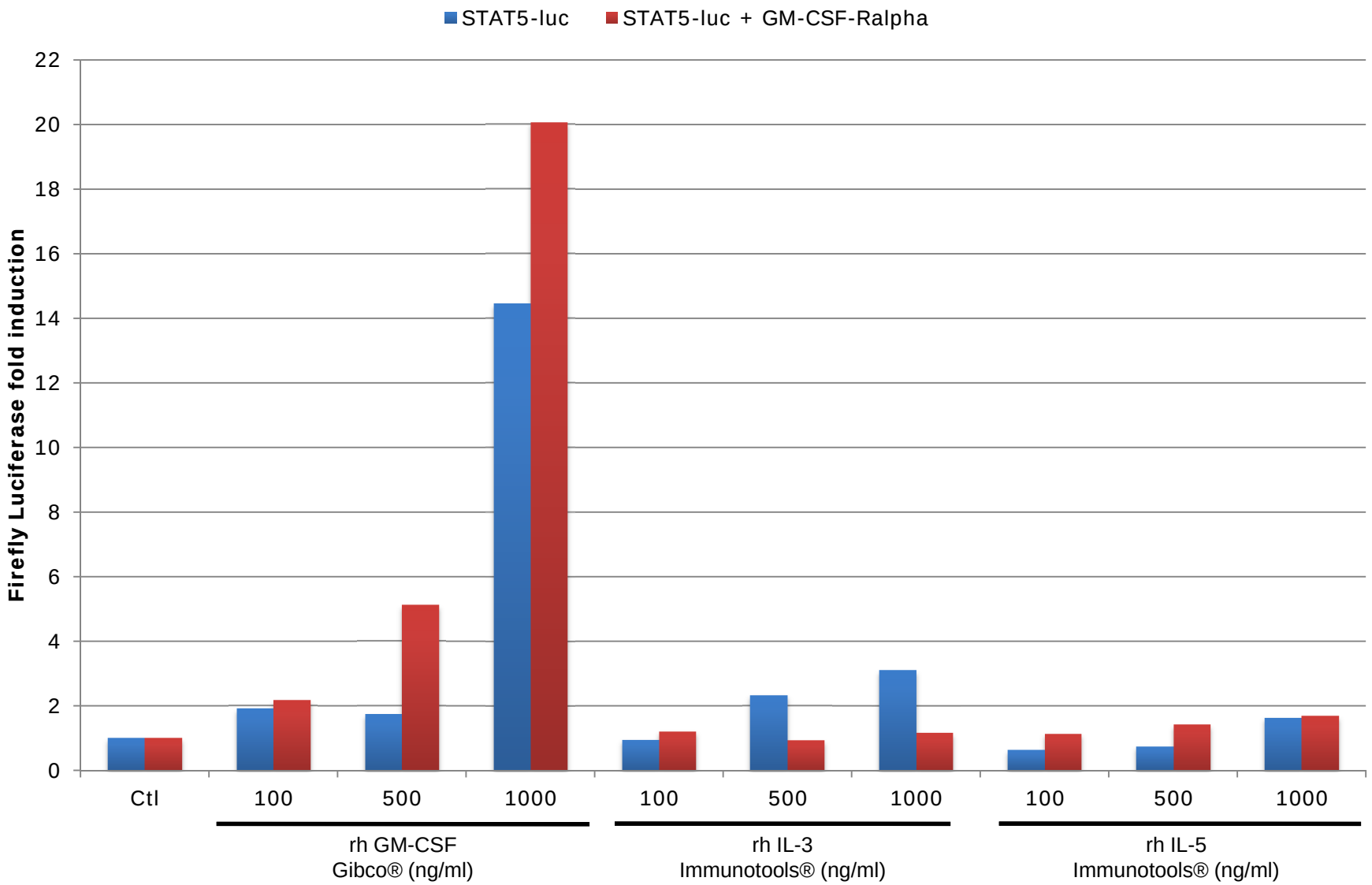
GM-CSF Gene Reporter Assay (FireFly / Renilla)



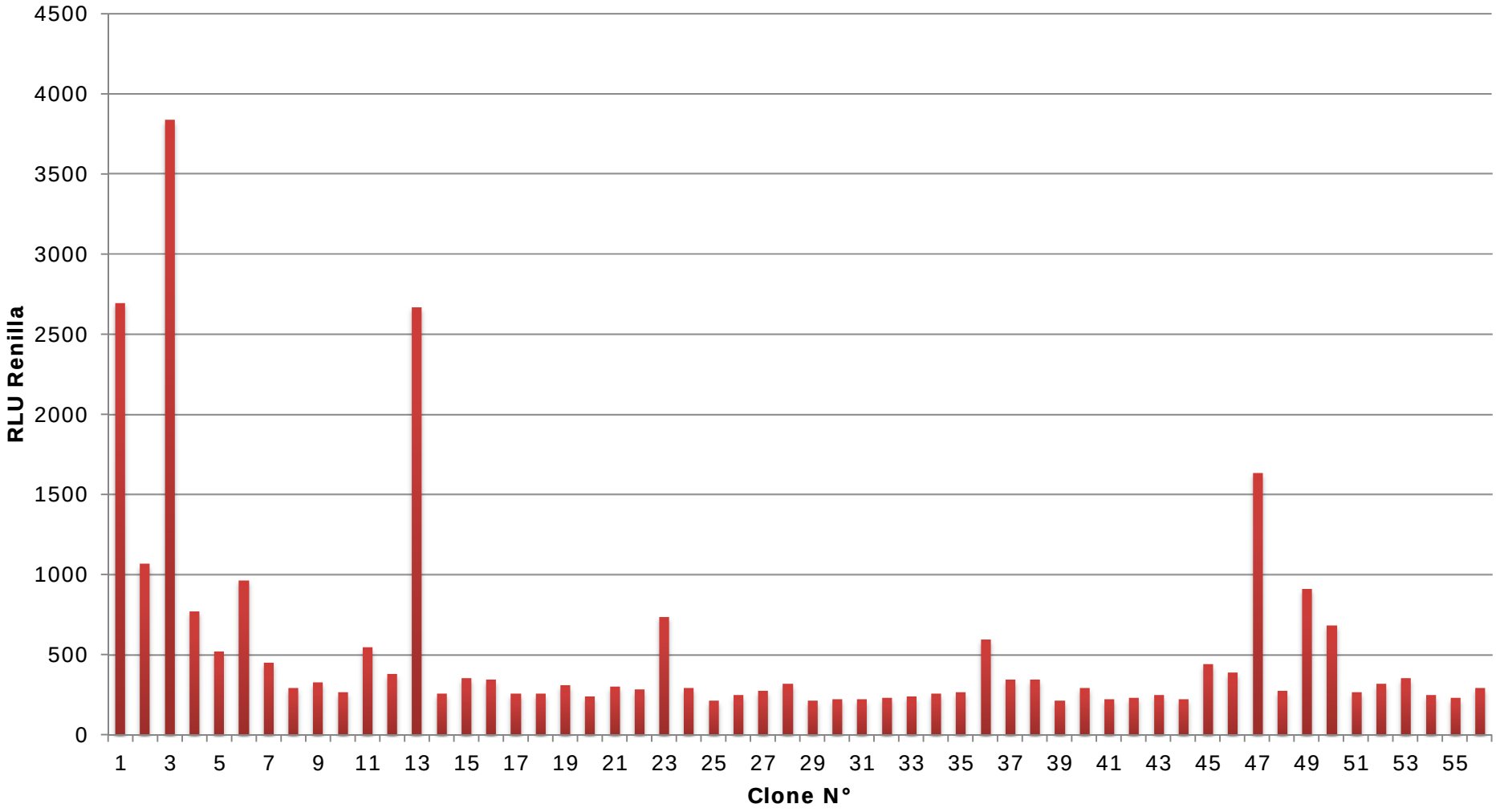
Double transcient transfection **STAT5**-luc (**C** or **D**) and pCMV-GM-CSF-Rα in U937 cells



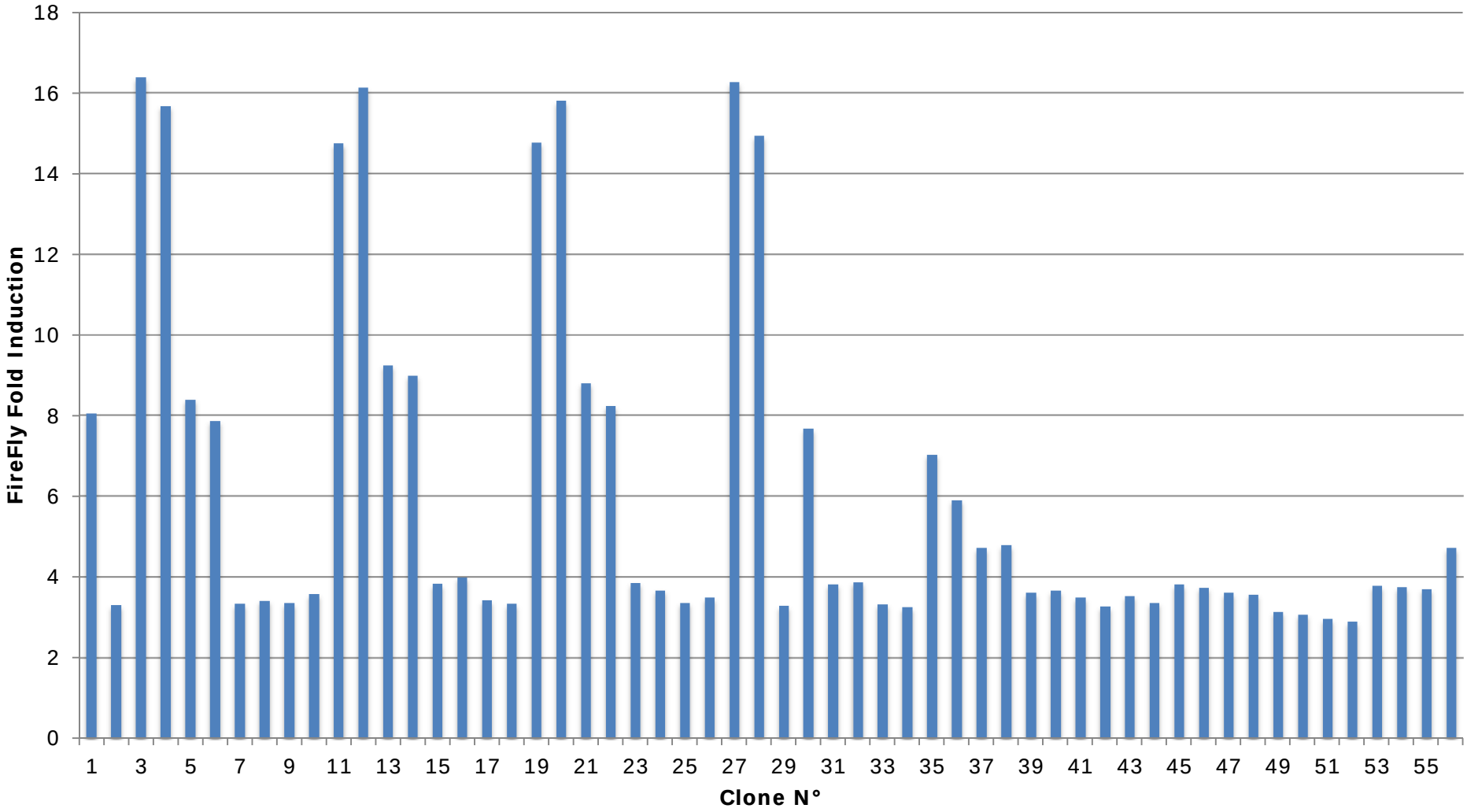
Firefly Luciferase induction in the U937 cell line *transciently tranfected* with STAT5-luc and GM-CSF-Rα treated with GM-CSF, IL-3 or IL-5



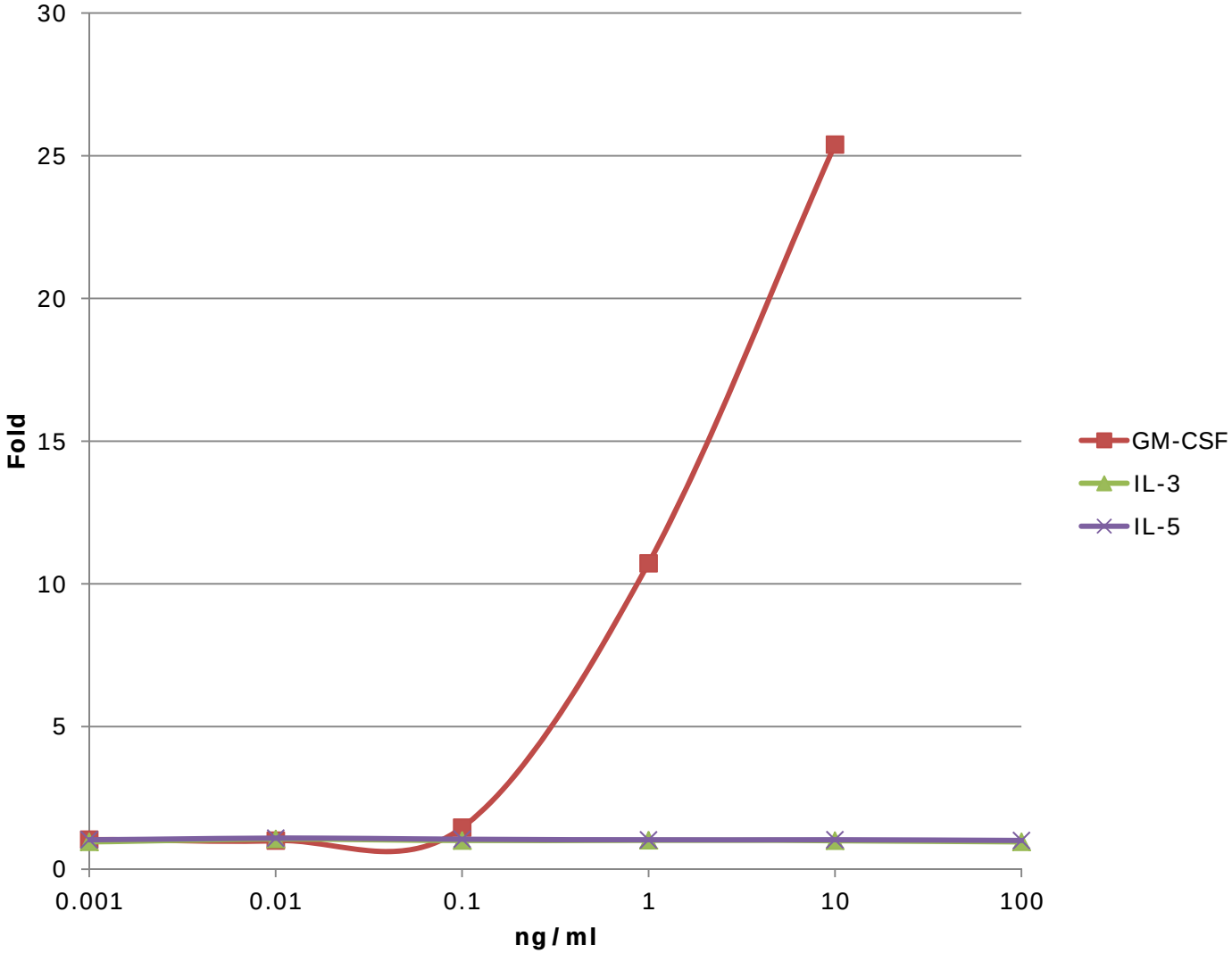
Analysis of US5-Luc Clones Expressing Renilla Luciferase and the GM-CSFRα Receptor Chain



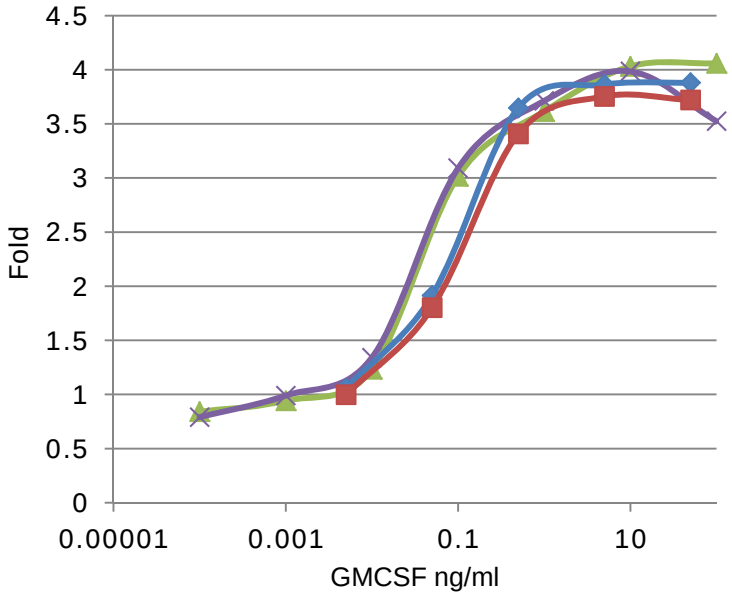
Analysis of RUS5-Luc clones Expressing Renilla Luciferase and GM-CSFα Receptor Chain



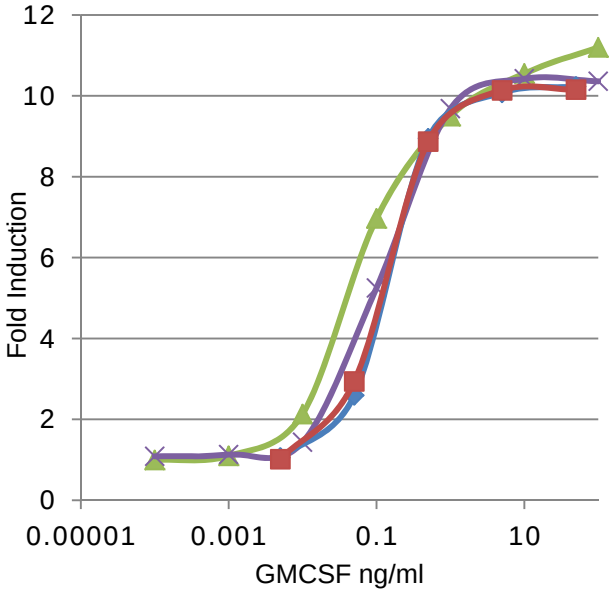
US5 Dose Response Curve (16h Incubation)



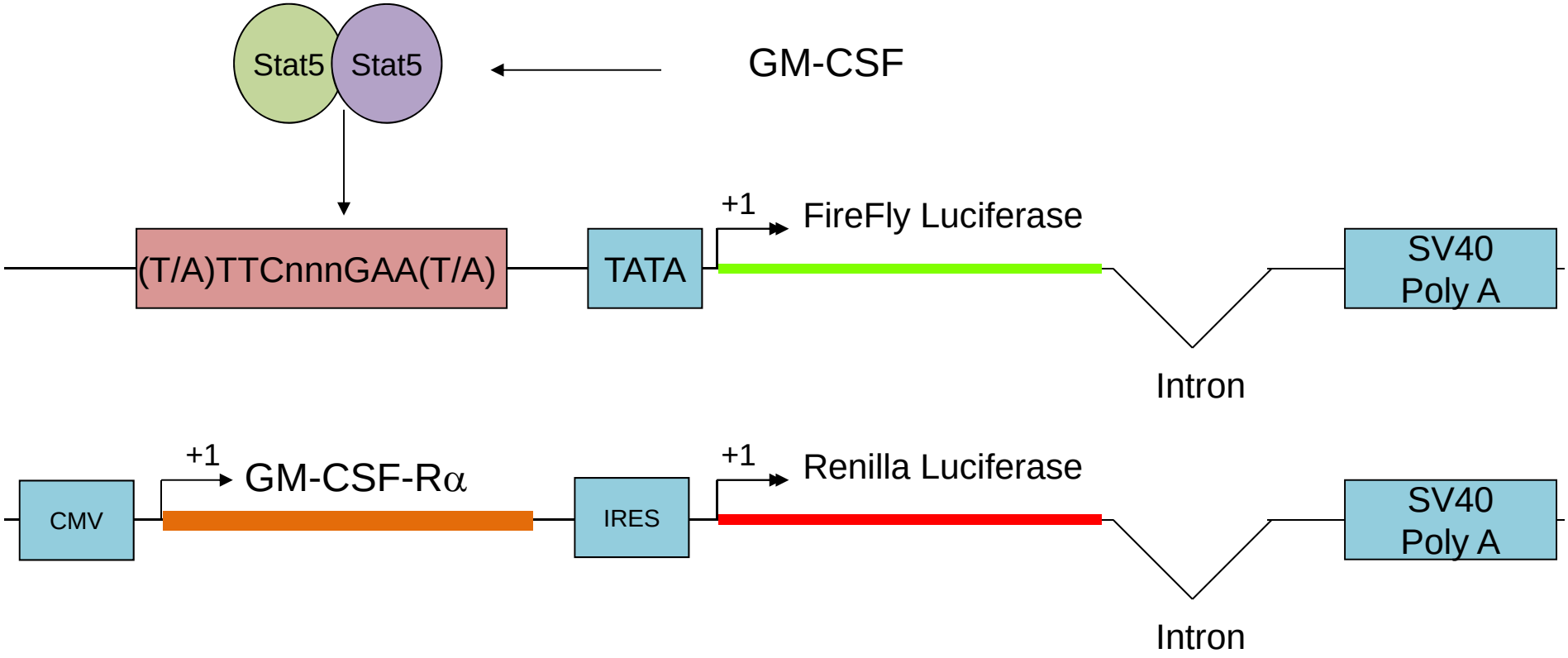
US5 GM-CSF (4h incubation)



US5 GM-CSF (16h incubation)

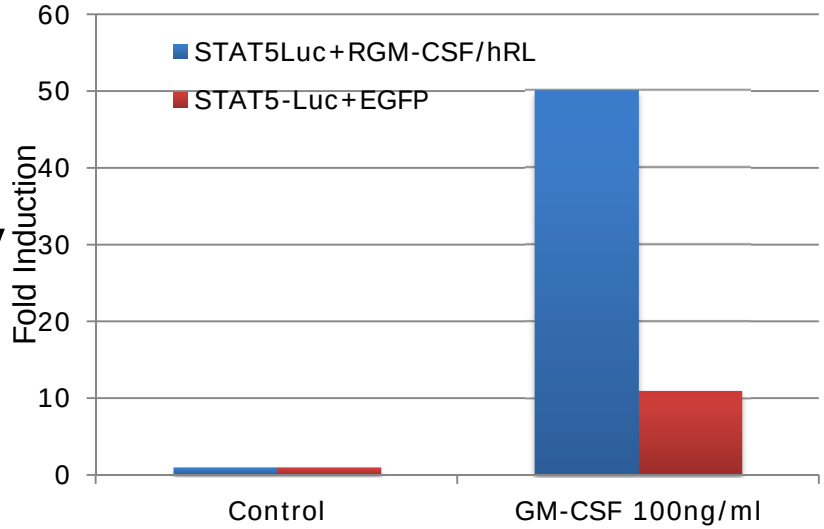


GM-CSF Gene Reporter Assay (FireFly / Renilla)

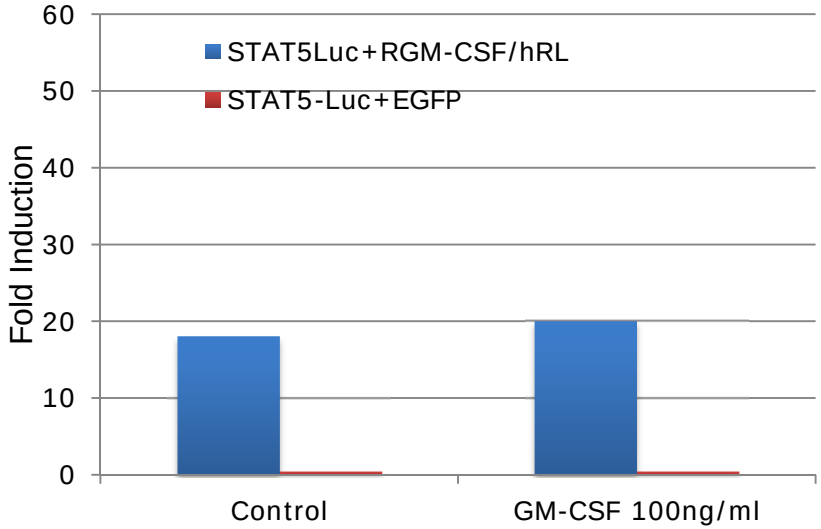


Transient Cotransfection of U937 Cells

FireFly Activity



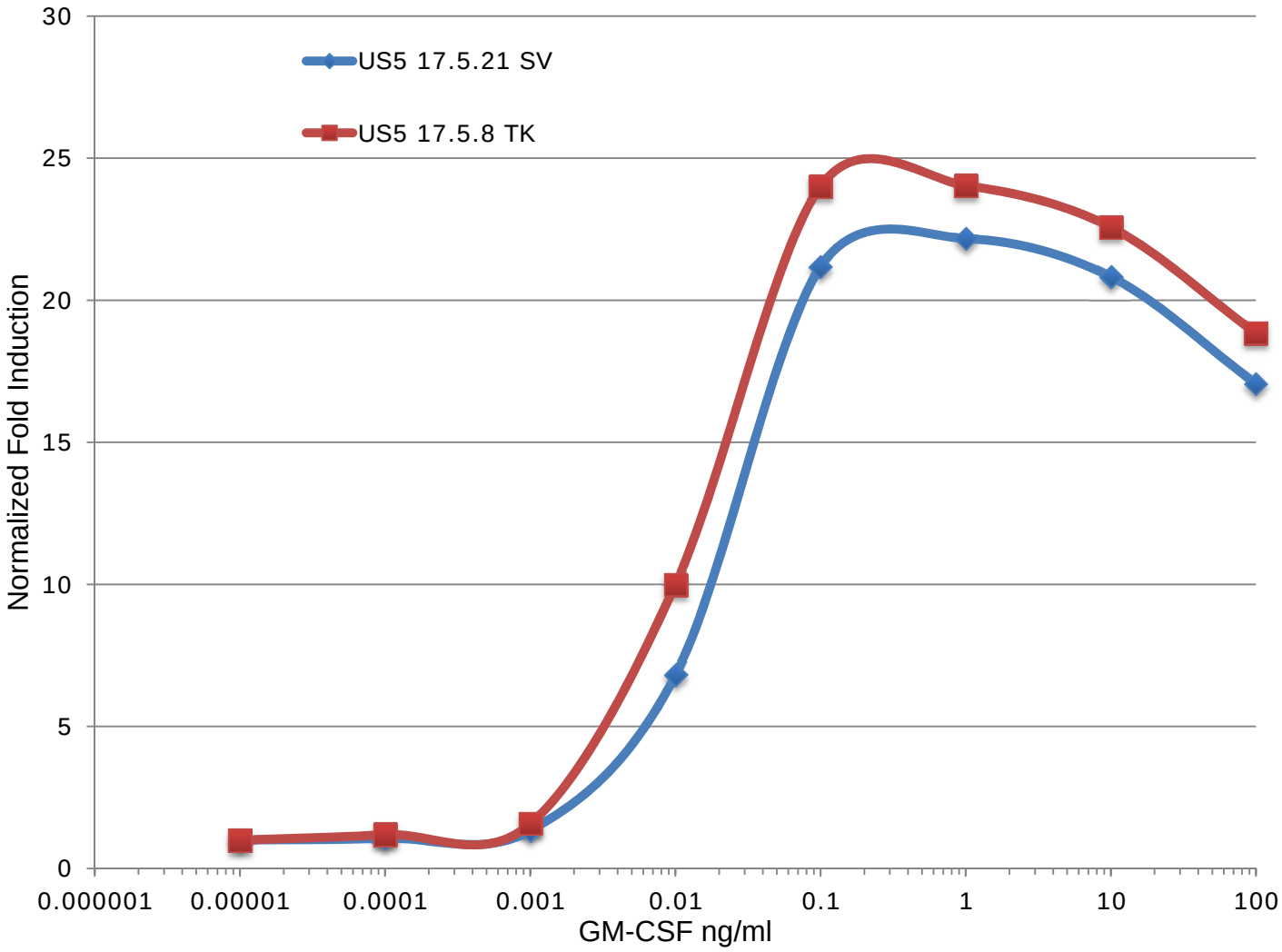
Renilla Activity



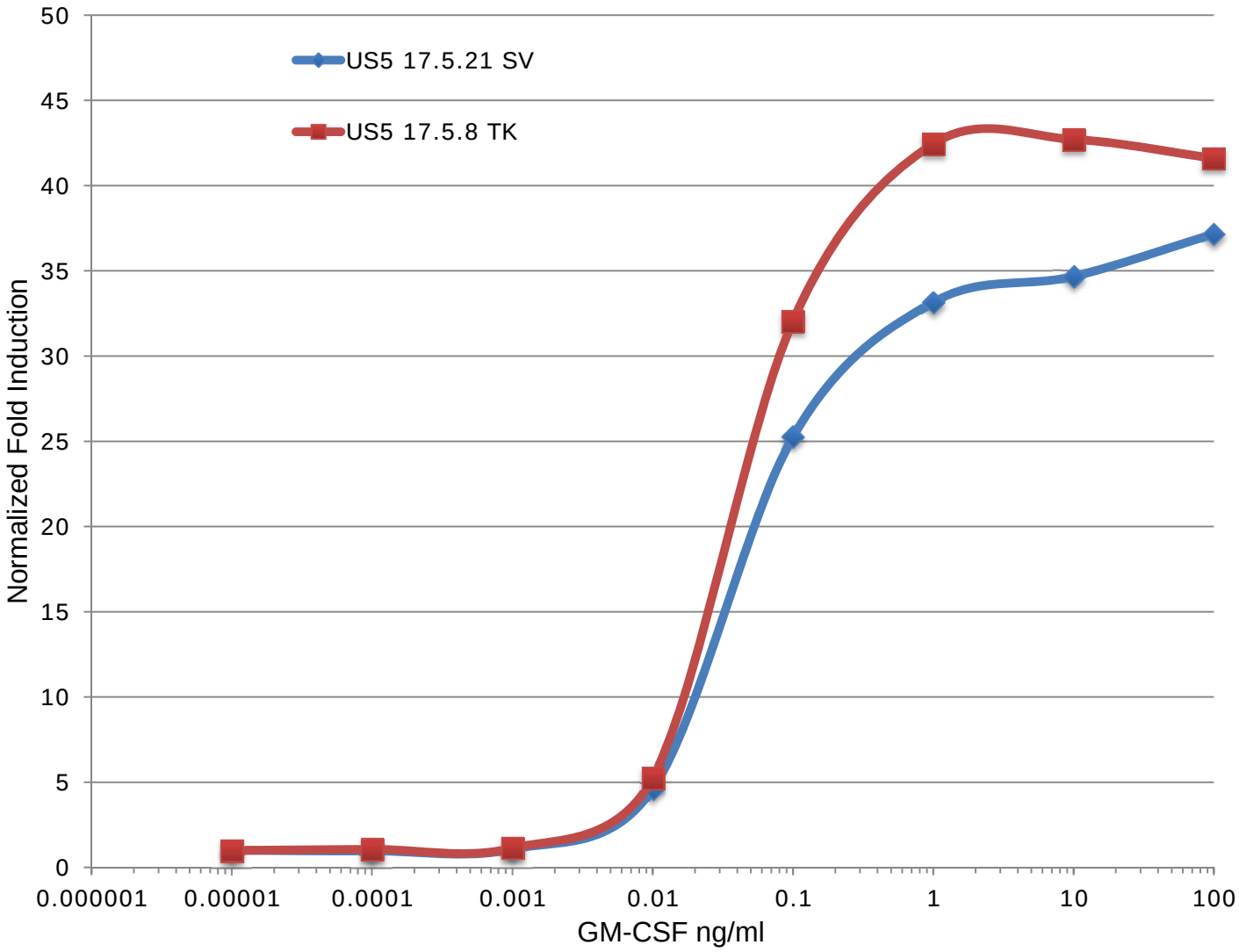
Normalized Reporter Gene Assay for GM-CSF

- *Unexpectedly,* constitutive expression of Renilla luciferase is influenced (2x) by STAT5 activation in response to GM-CSF treatment
- Consequence effective dynamic range of assay reduced
- Mechanism unclear, no Stat5 recognition sequences in promoter construct
- *Solution* change promoter use SV40 constitutive promoter & Herpes simplex TK promoter
- Use humanized Gaussia luciferase gene instead of Renilla luciferase

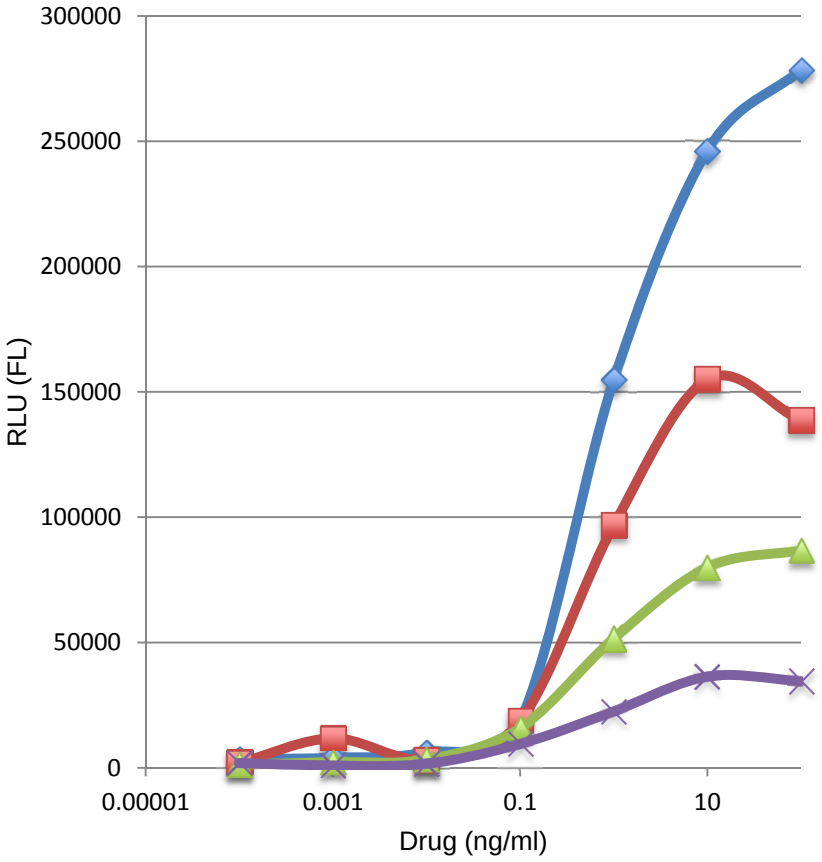
GM-CSF Induced FL Expression Normalized Relative to Gaussia Expression (4 H Induction)

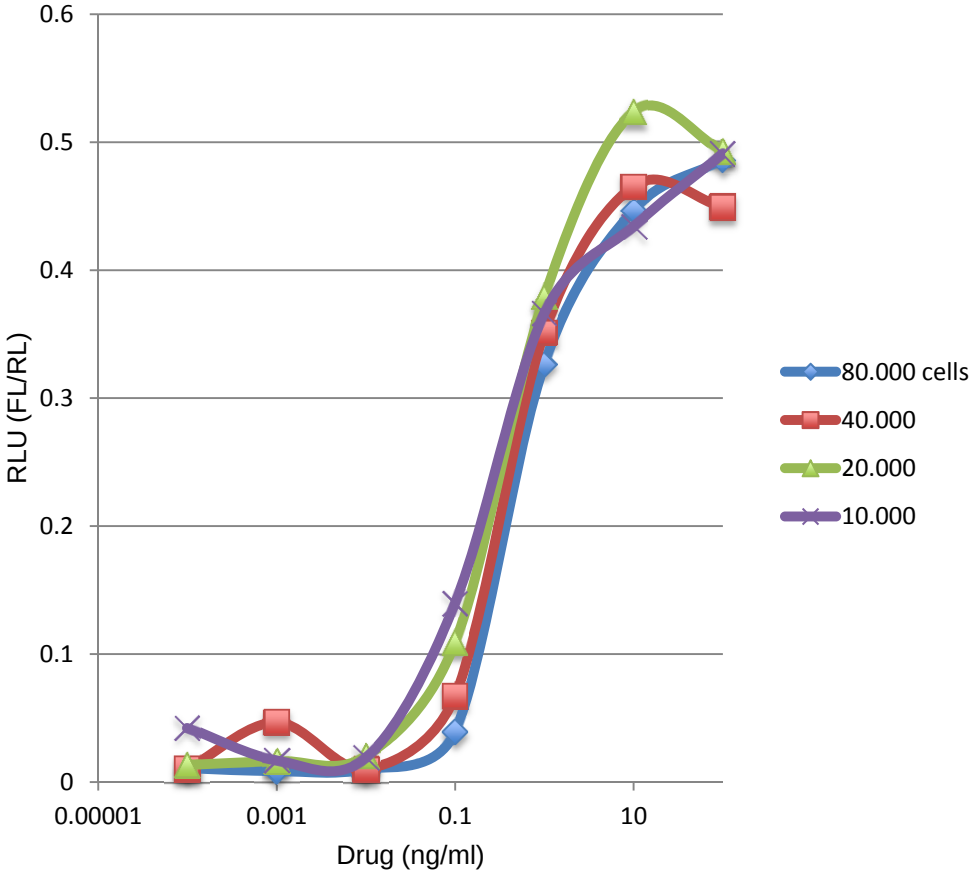


GM-CSF Induced FL Expression Normalized Relative to Gaussia Expression (16 H Induction)

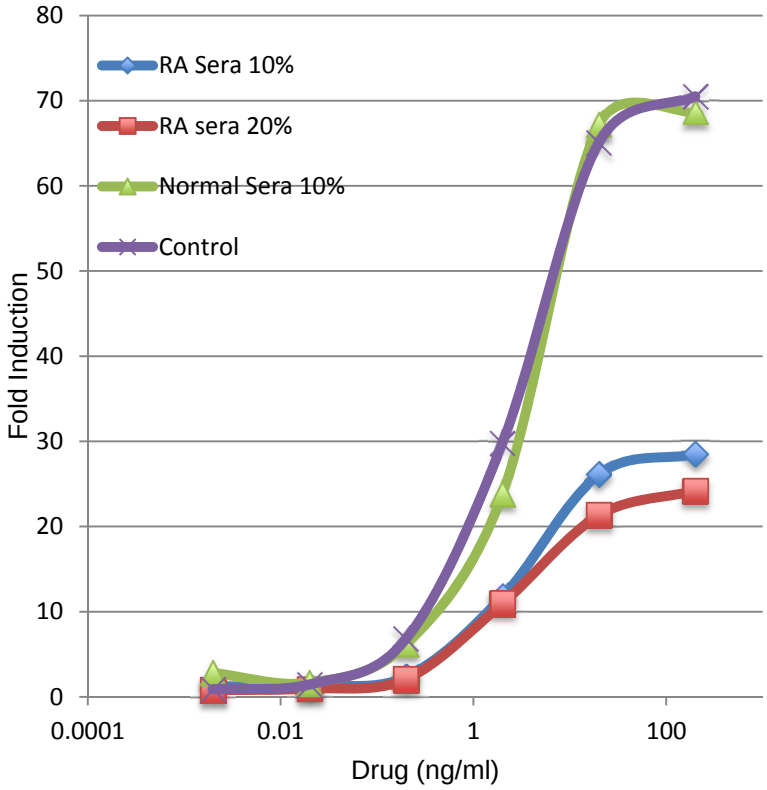


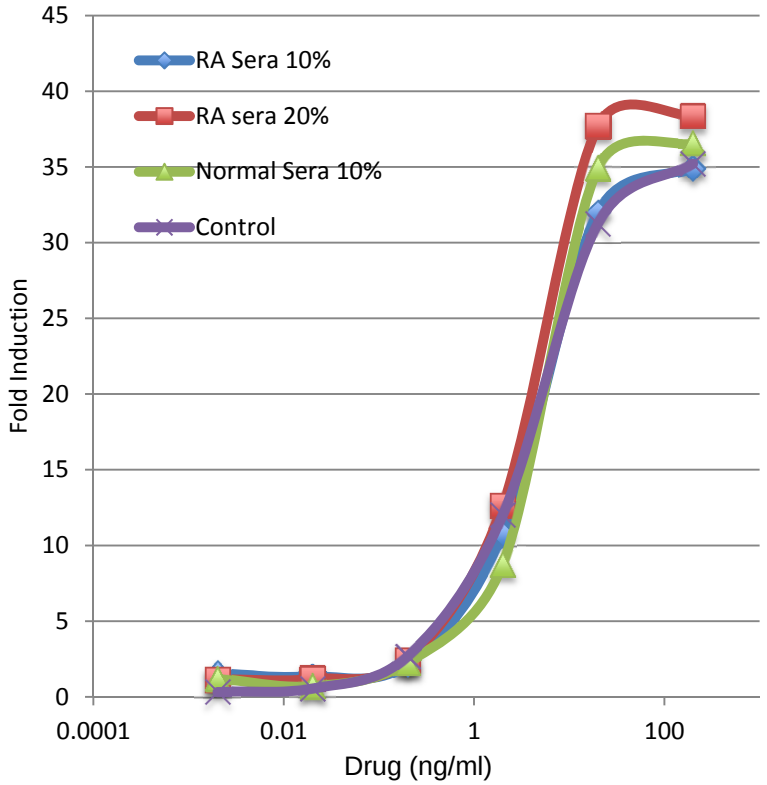
Relationship Between Drug Induced FL Expression and Cell Number





Response of Reporter Cells to Serum Matrix Effects





Stability Studies: Sensitivity

Time (Hrs)	Passage #	EC50 (pg/ml)	LLOQ (pg/ml)
4	10	40	10
4	20	80	20
4	40	20	5
18	10	400	200
18	20	400	200
18	40	200	100

Stability Studies: Proliferation

Passage #	Doubling Time (Hrs)	Max Cell Density ($x10^6$/ml)
4	27	1.0
10	25	0.95
20	24	1.2
40	24	1.1

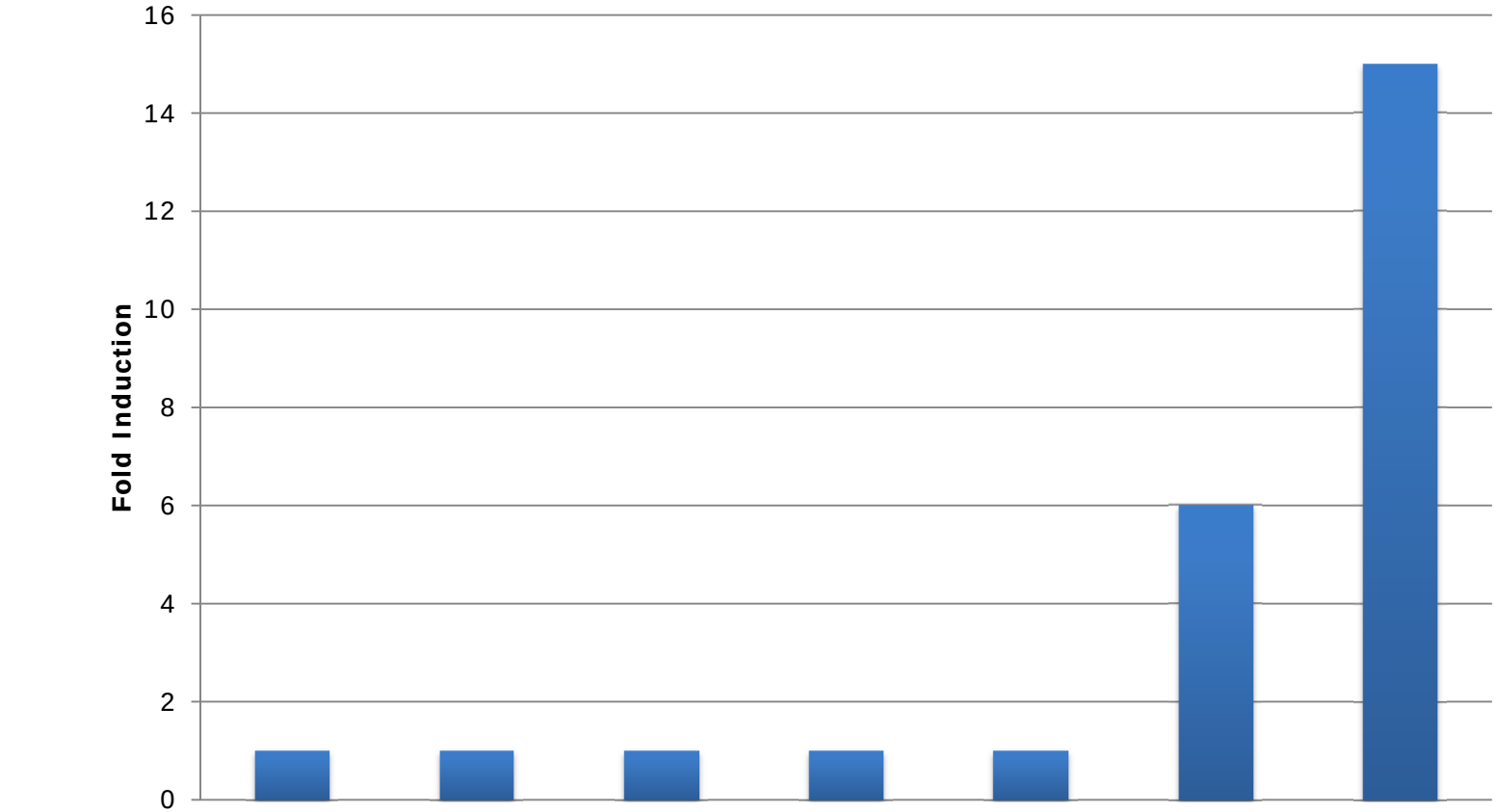
Conclusions - I

- A cell-based assay specific for a growth factor can be established by the use of a cell-line that does not require the growth factor or other related grow factors for proliferation
- The cell does, however, contain a functional receptor/signal transduction system for the growth factor of interest
- The assay can be rendered specific by over-expression of a growth factor specific binding receptor sub-unit

Conclusions - II

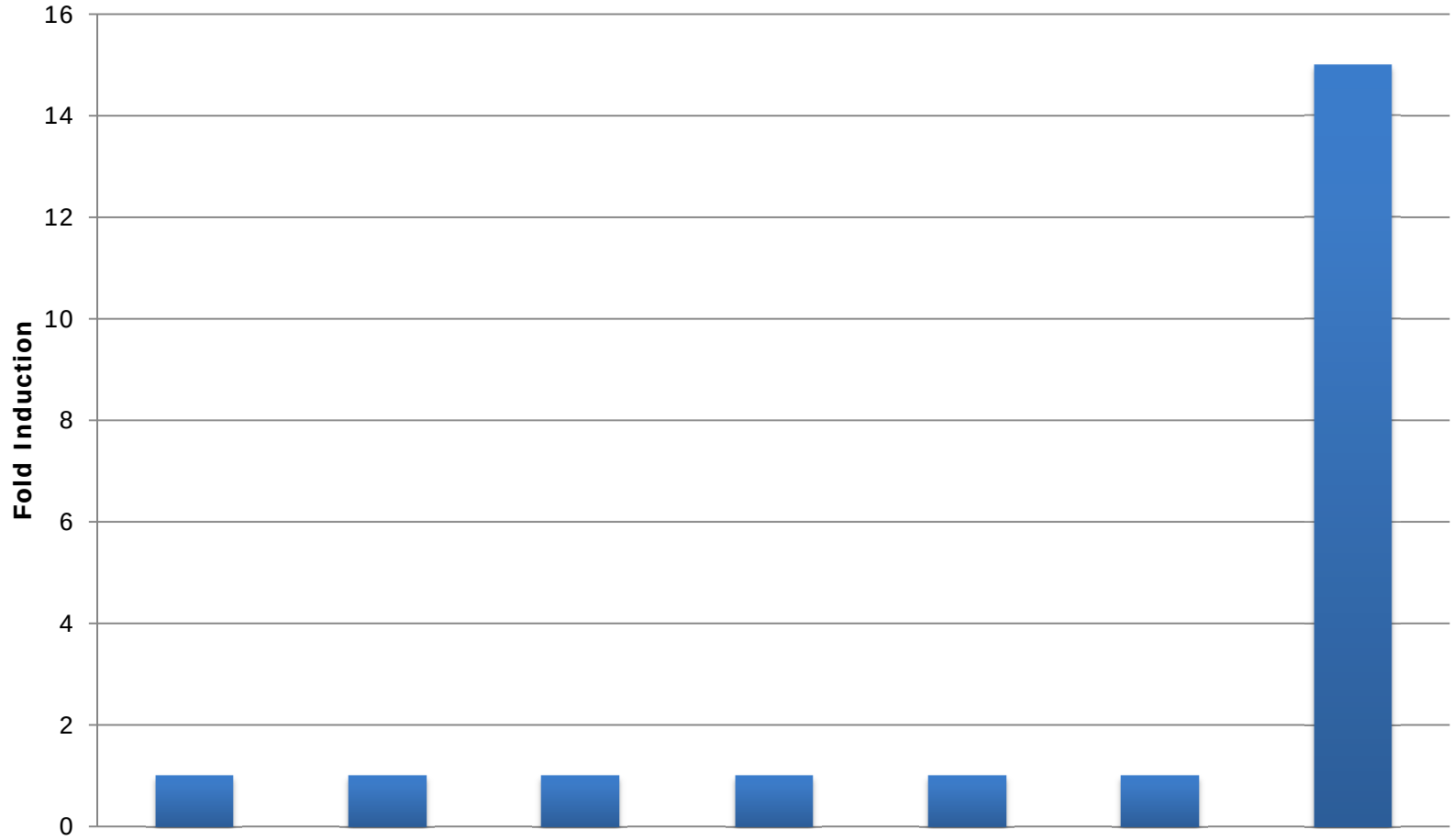
- When a growth factor signals through multiple pathways (MAPK, NFkB, $STAT_{1-5}$ etc) it may be more useful to reconstitute a *complete* receptor-signaling transduction system in a cell that does not respond to the cytokine of interest.

Reconstitution of a Functional Cytokine Signaling Pathway in Human U937 cells



Stat4-kozak	-	+	-	-	+	-	+
CX2RB1	-	-	+	-	-	+	+
CX2RB2	-	-	-	+	+	+	+
STAT4-Luc	+	+	+	+	+	+	+

Reconstitution of a Functional Cytokine Signaling Pathway in Human HEK cells



Stat4-kozak	-	+	-	-	+	-	+
CX2RB1	-	-	+	-	-	+	+
CX2RB2	-	-	-	+	+	+	+
STAT4-luc	+	+	+	+	+	+	+

Conclusions - III

- Reconstitution of a cellular receptor-signal transduction system in a cell that does not respond to the cytokine or growth factor of interest is a powerful tool for the establishment of Specific cell-based assays for the quantification of the activity and neutralizing antibody response to therapeutic proteins

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