



Multiplexed Transcription Factor Profiling for Post-Genomic Studies

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Assays and Cellular Targets

November 1, 2006

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Questions of the Post-Genomic Era

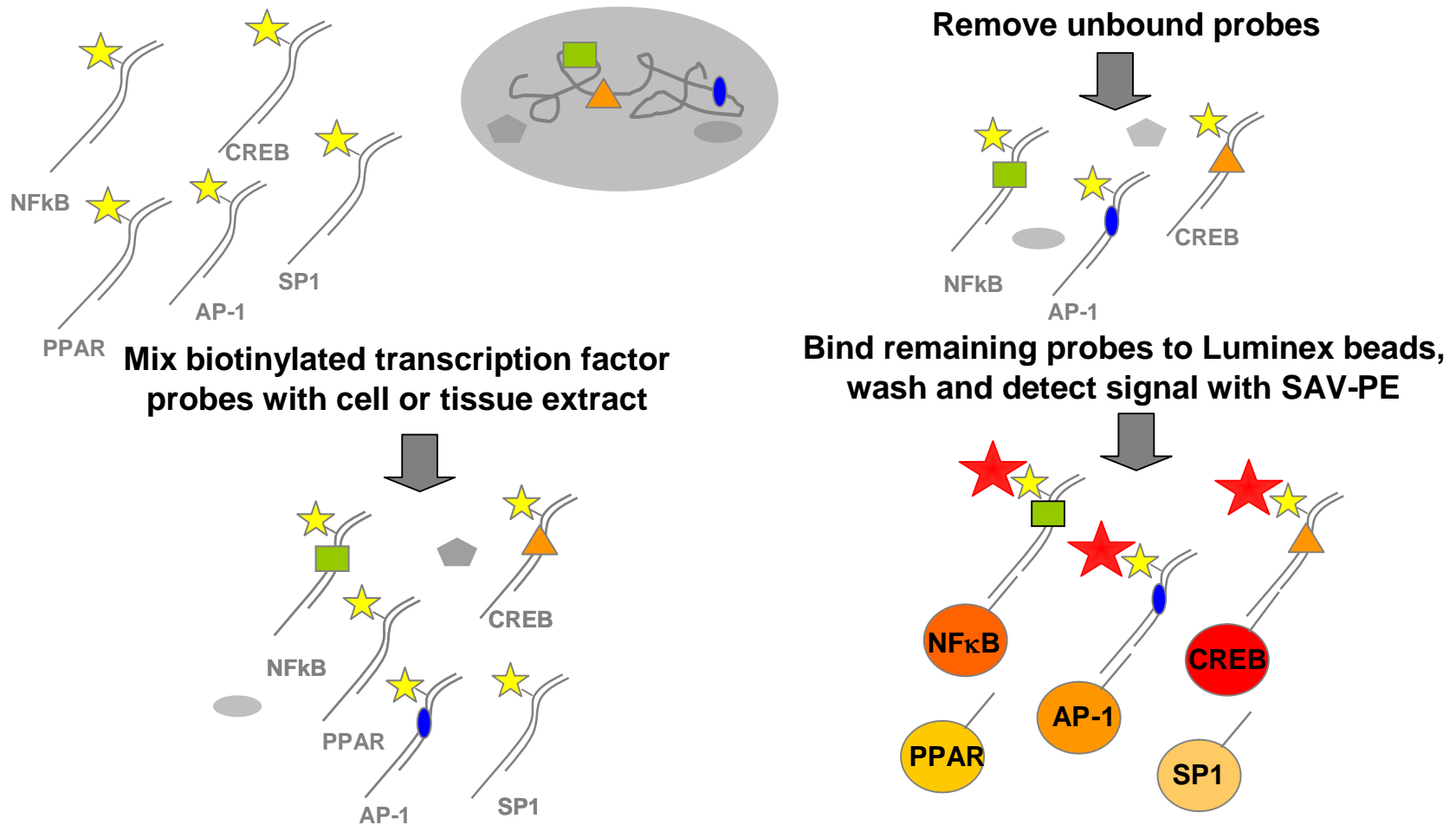
- From an external stimulus, what series of molecular events leads to a phenotypic change?
- Given that we can accurately measure gene expression, can we now identify upstream signaling events that control the cellular response?
- Can these events be controlled, targeted or modified?
- Can gene expression data be combined with protein-DNA interaction and proteomics data to provide a complete picture of the cellular state?



Transcription Factor Profiling Technology Overview

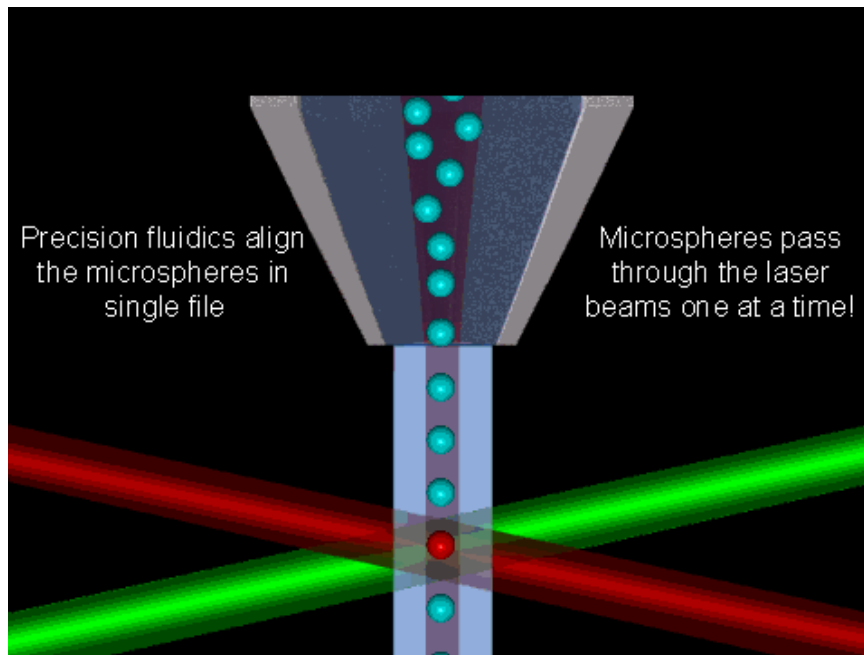


How the Multiplex Assay Works



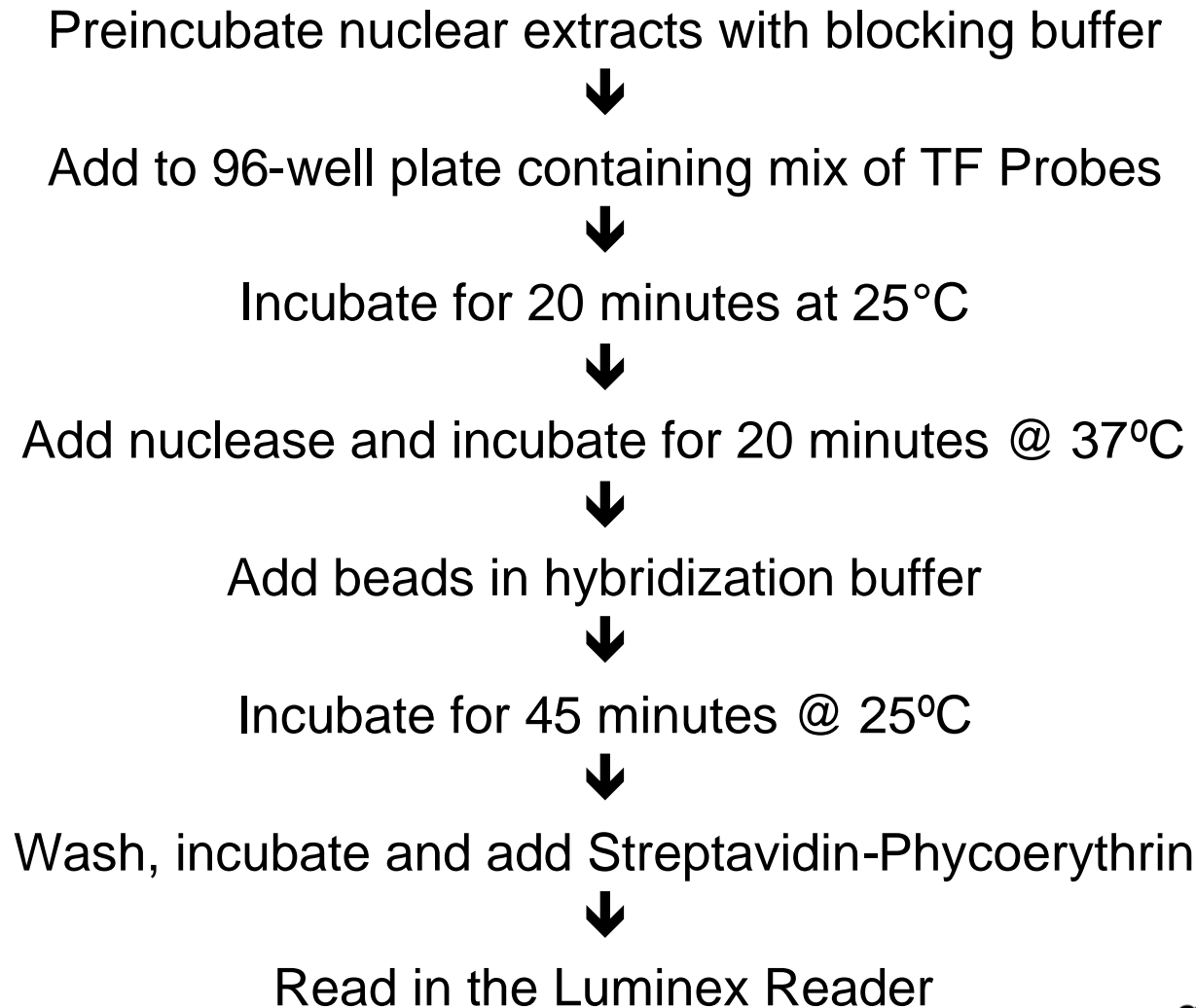
Total Assay Time = 120 minutes
Reading Time = 60 minutes

DNA Binding is Detected by Luminex Reader



- The different DNA-binding activities are distinguished via different colored beads
- PE fluorescent intensity quantifies DNA-binding activity

Assay Workflow



Total Assay Time = 120 minutes

Reading Time = 60 minutes



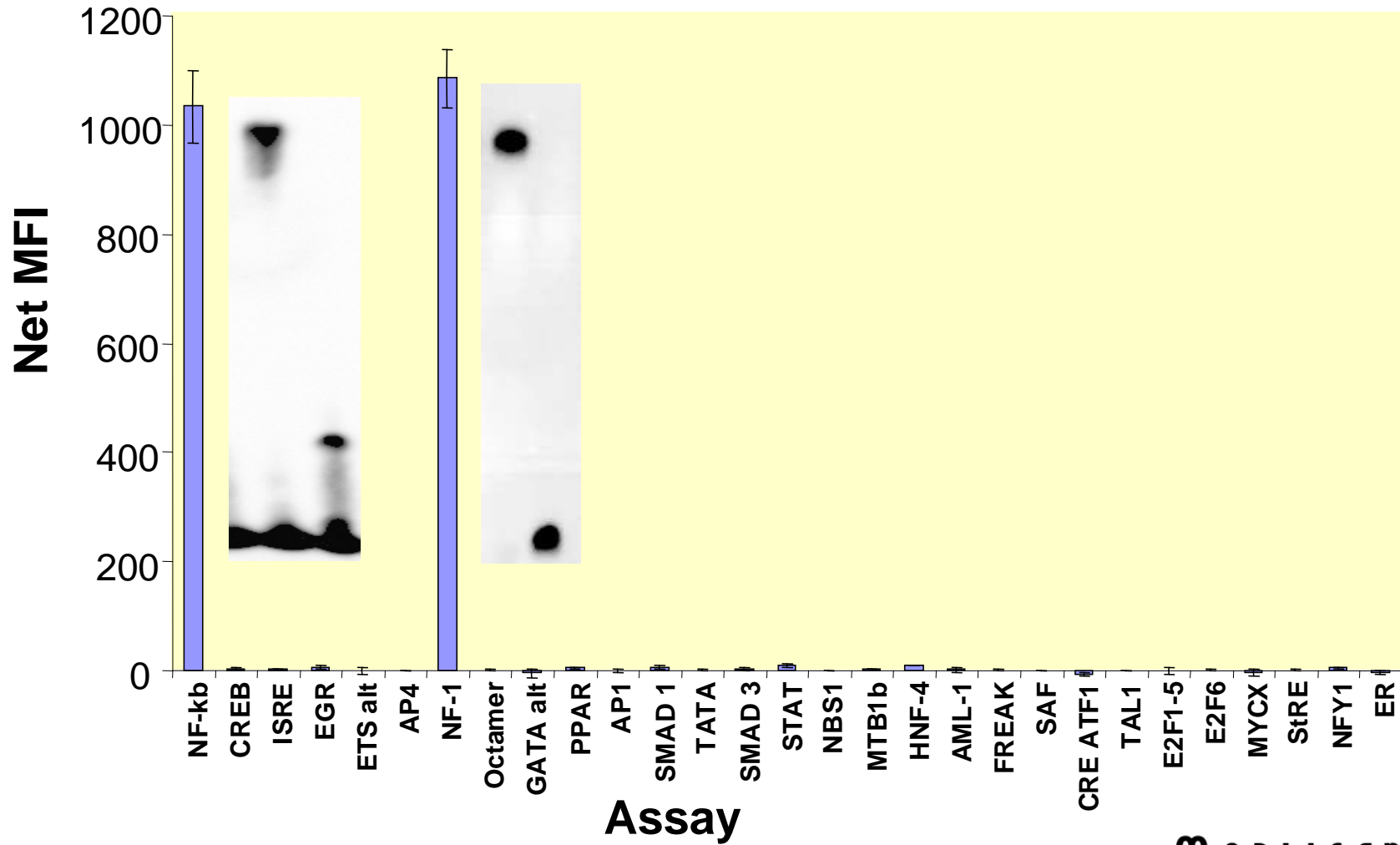
Assay Validation and Performance



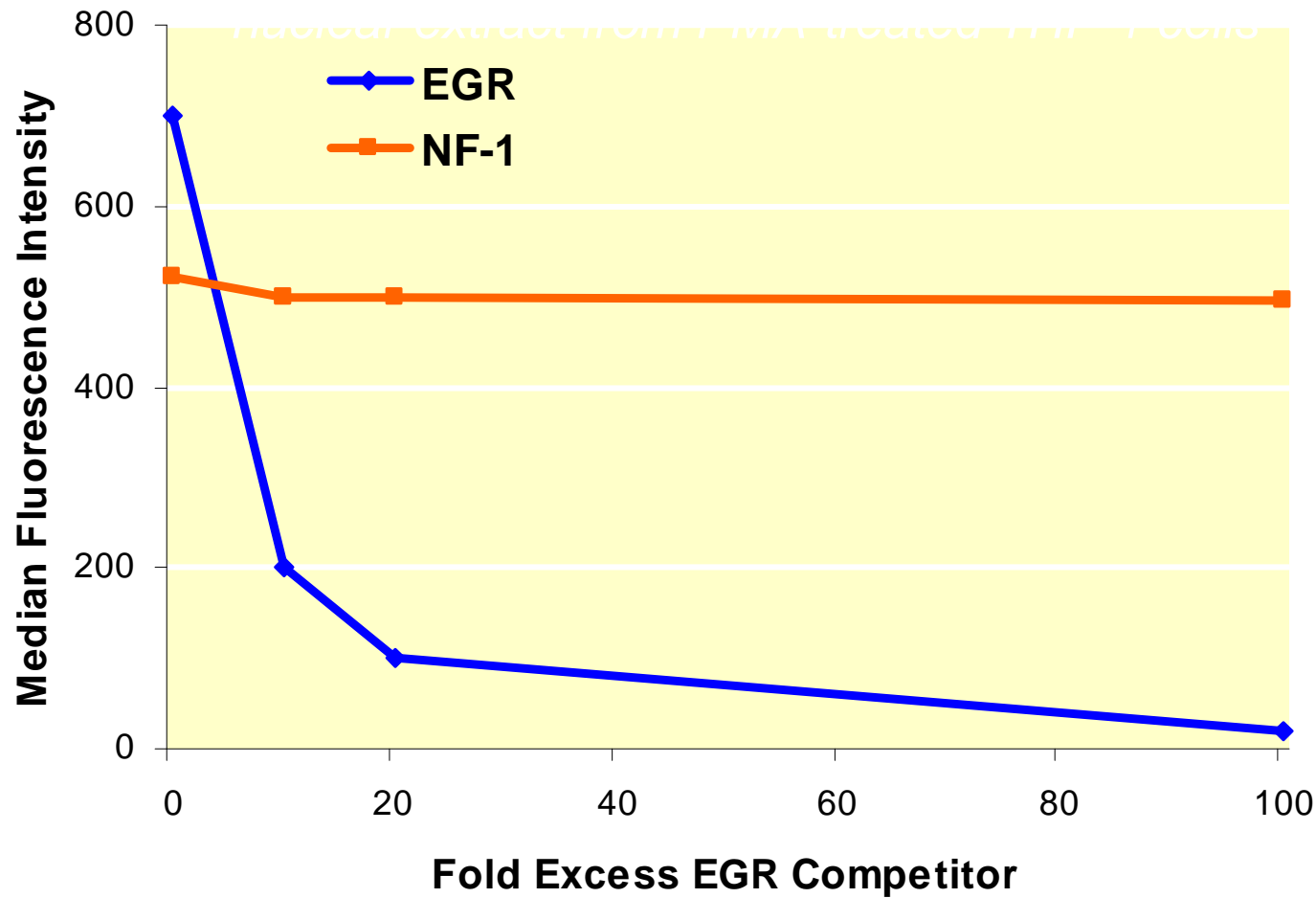
Multiplex Assay Validation and Performance

- Validation
 - Bioinformatics
 - Pure protein studies
 - Transfection studies
 - Model Systems
 - Competition assays
- Cultured Cells
 - Nuclear extracts from cell lines and primary cells
 - Nuclear extracts from tissues (liver, brain, cardiac muscle)
- Animal models and tissues
 - High homology of binding sites predicts that assay should work from yeast to man
 - Verified in human, mouse, rat, mink, monkey

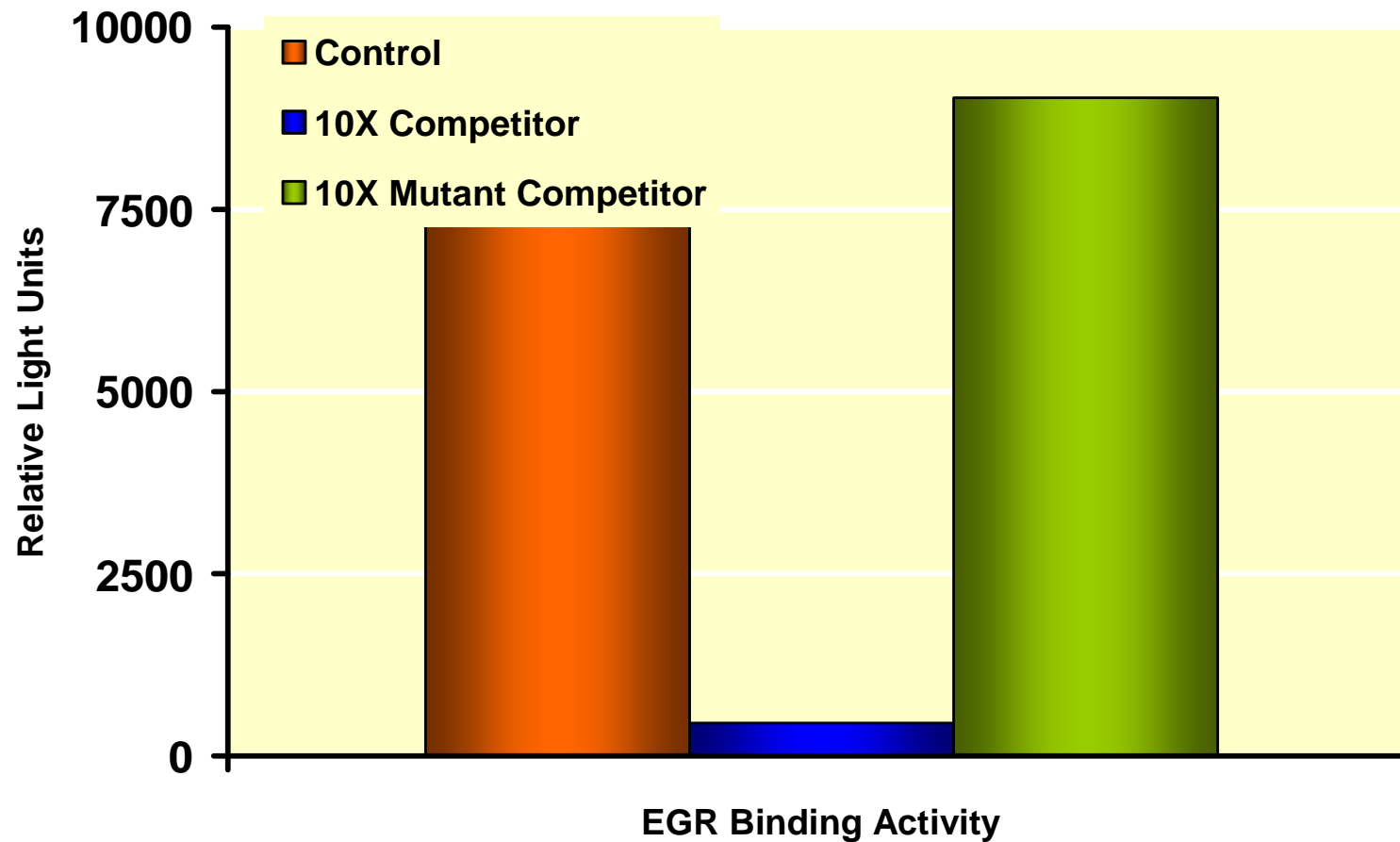
Transcription Factor Profile Obtained with Recombinant NF-kB p50 and NF-1



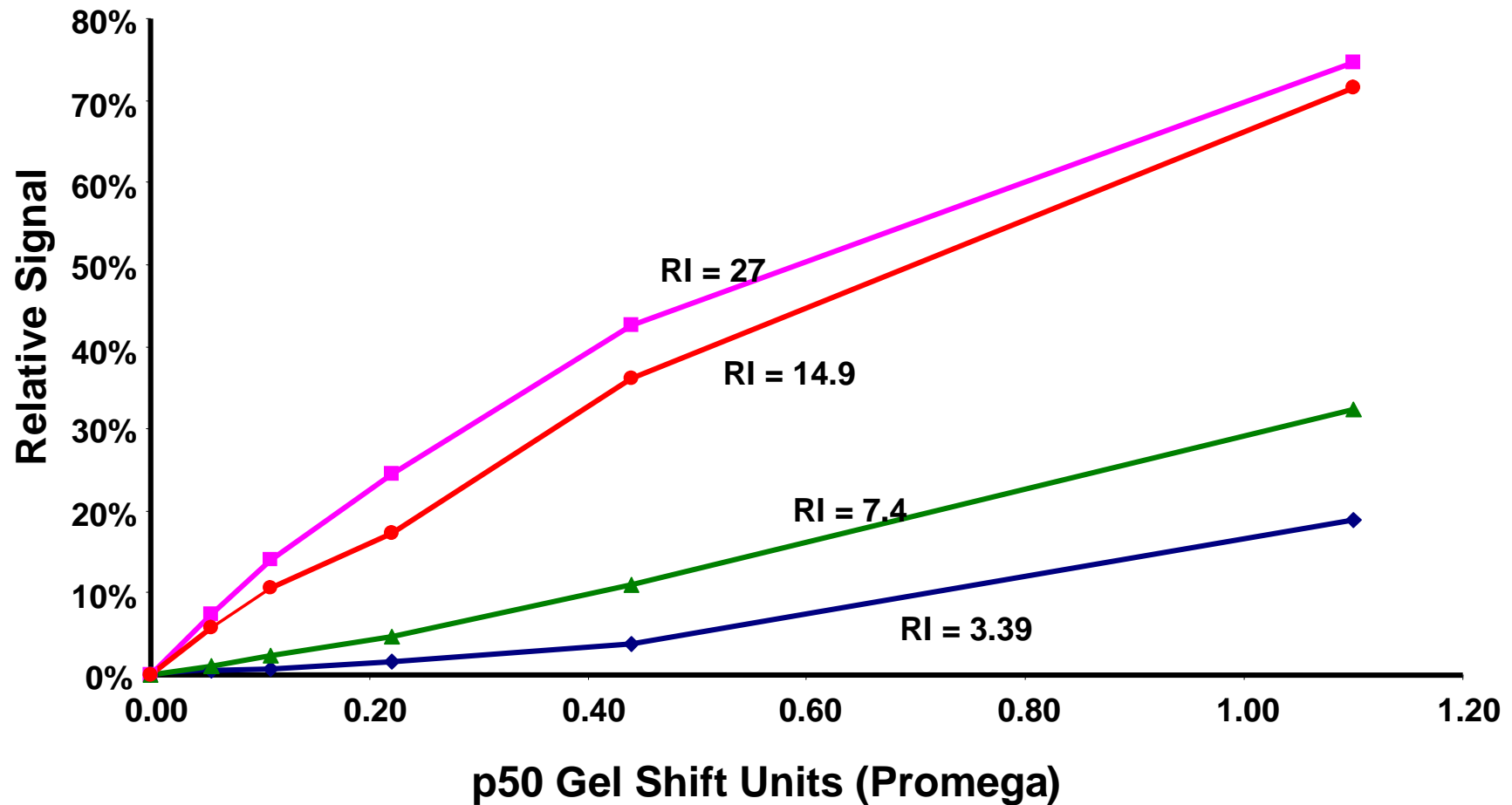
Specificity of Binding Is Demonstrated by Competitive Binding Assays



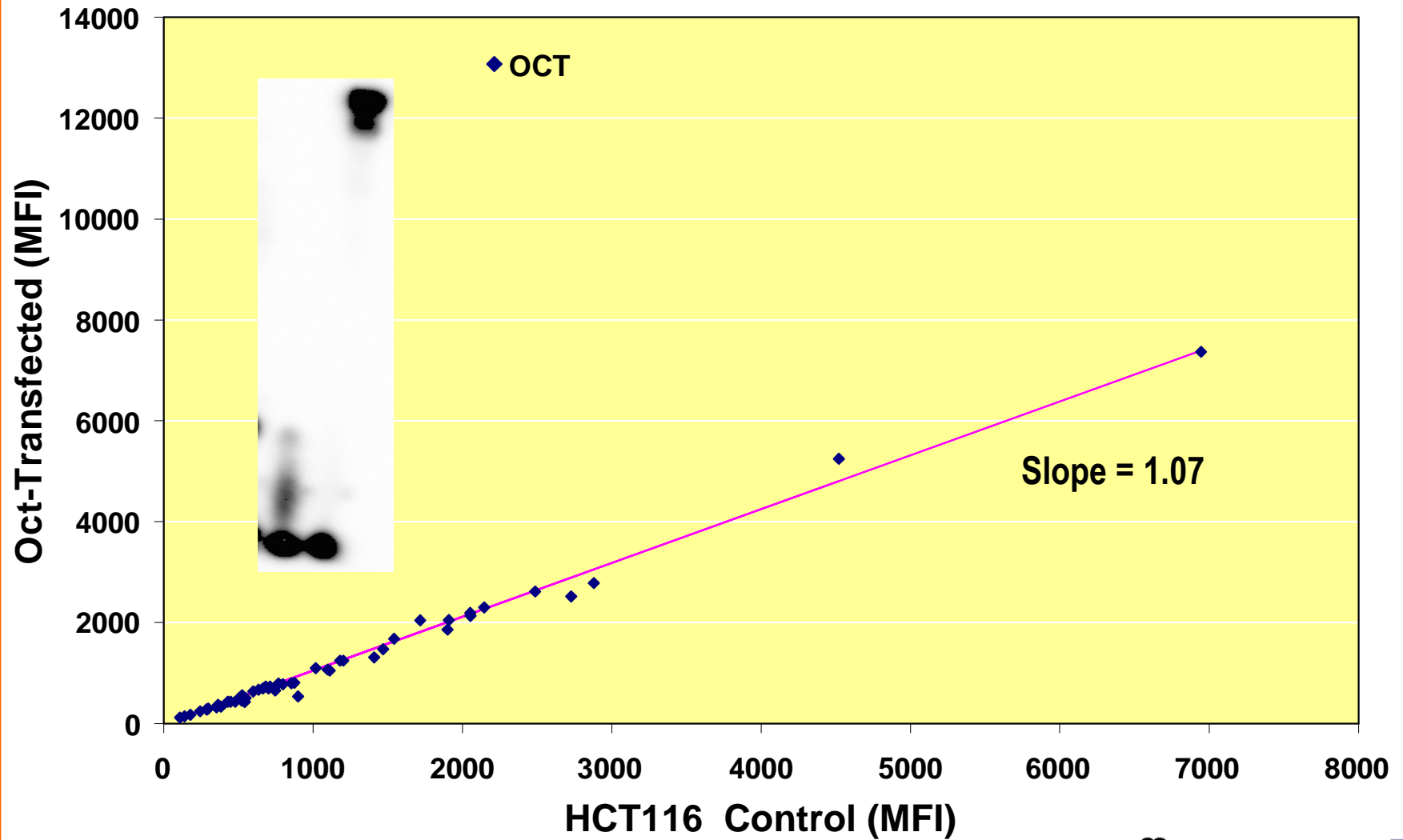
Demonstration of Specificity by Competitive Binding



Analysis of Binding Sites with Different Predicted affinity for NF-kB p50 Protein



Transfection Analysis

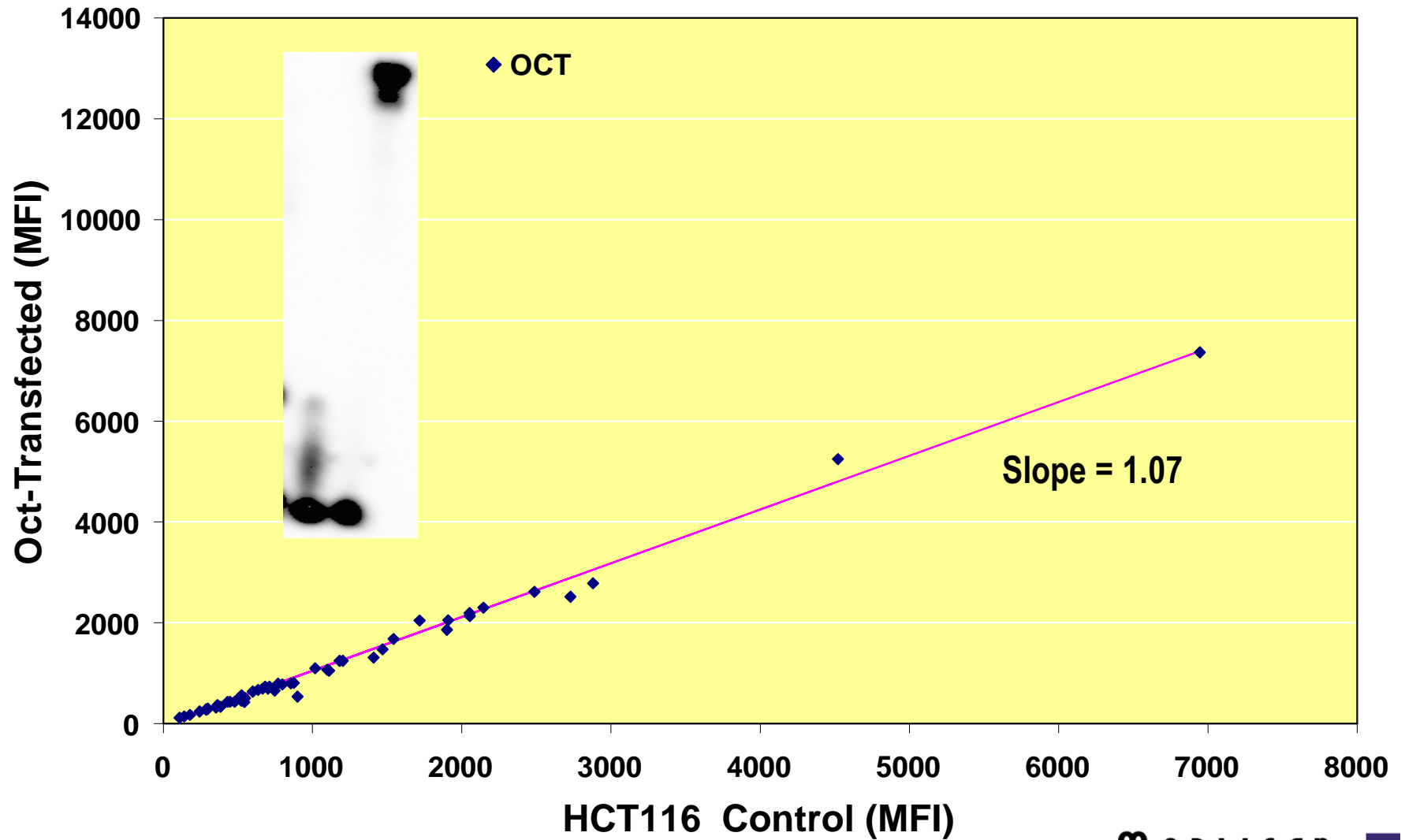


	A	B	C	D	E	F	G	H	I
1	SampleID	HCT116 6-12	Oct	Y Predicted	Diff Sqrd	% from Predicted		Oct vs HCT116 6-12	
2	Oct	2214	13073	2346	115069233	457%			
3	PBX	108	117	99	321	18%		Set Fitting Parameters	
4	NFkb	1717	2047	1815	53654	13%		OLS Slope	1.23
5	PPAR	4520	5250	4806	197236	9%		OLS Intercept	-36.06
6	ER	140	141	133	60	6%			
7	SRE	524	564	543	430	4%		LMS Parameter	829
8	P53	682	736			3%			
9	MEF2	1543	1679			3%		Enter Predicted Values Here	
10	C-MYB	1018	1098			3%		Predicted Slope	1.07
11	CEBPg	600	634			2%		Predicted Intercept	-16.49
12	STAT GRR	634	671			2%			
13	E2F6	1909	2049			1%		Set Threshold Values	
14	HNF1	2146	2300			1%		Condition	
15	E2F1-5	2052	2192			1%		Low signal	50
16	SAF1	1180	1248			0%		% Increase	25%
17	TRE-AP1	664	693			0%		% Decrease	-25%
18	CEBPd	179	174	174	0	0%		LMS Fit	
19	AP4	362	370	370	0	0%			
20	NF1	6946	7365	7394	829	0%		Solver Fit	
21	CEBPα	243	242	243	1	0%			
22	NFAT	2486	2616	2636	393	-1%			
23	AML1	768	793	803	91	-1%			
24	SMAD 2-3	712	734	743	83	-1%			
25	ETS	1202	1245	1266	453	-2%			
26	SP1	2053	2134	2174	1561	-2%			

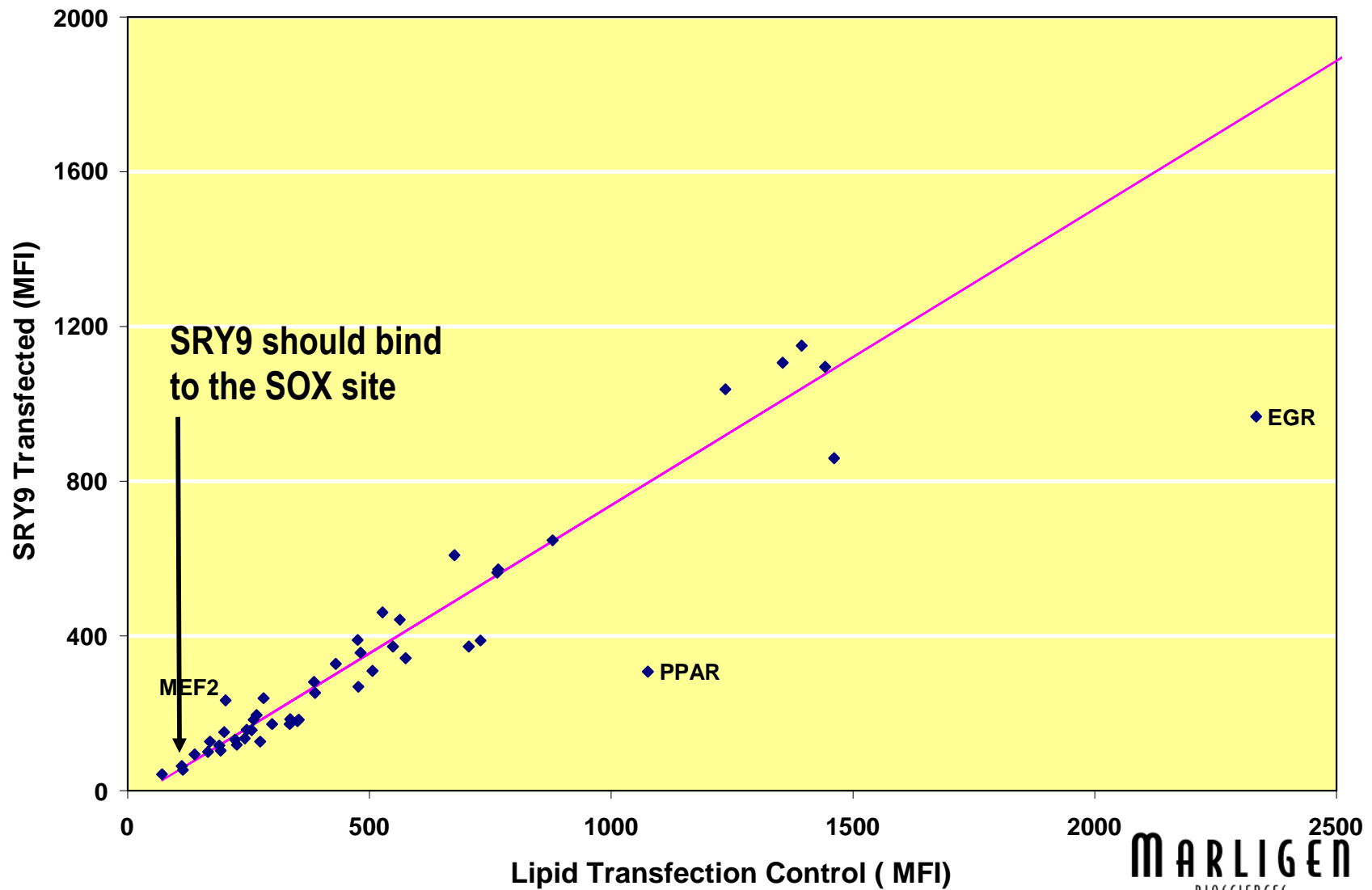
Marligen [X]

- Copy Sheet to End
- Copy Data to Last Sheet
- Rename Sheet to H1
- Reset Graph Ranges
- Sort by % from Pred
- Sort by % from Pred
- Sort by X MFI
- Sort by X MFI

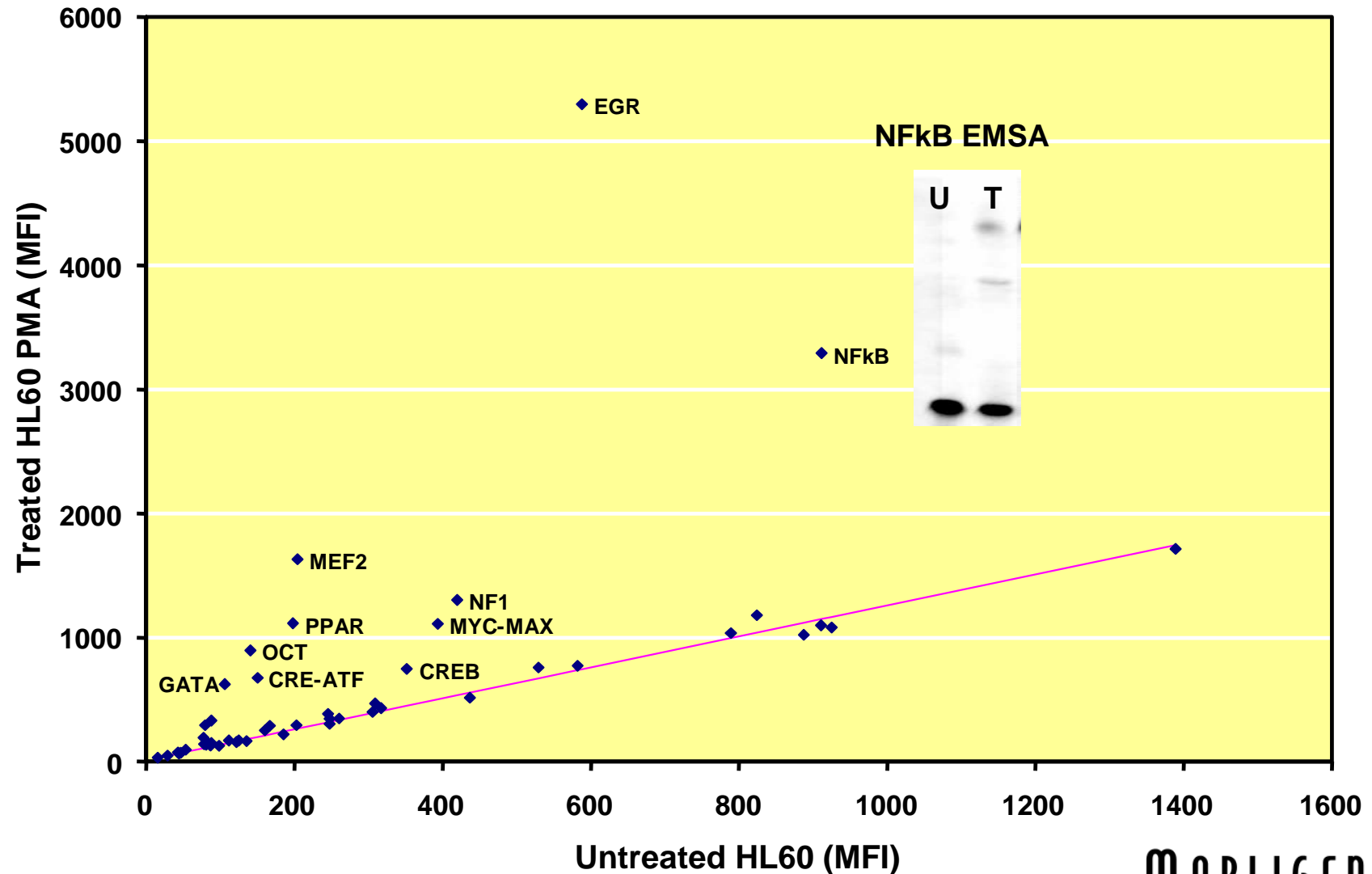
Transfection Analysis



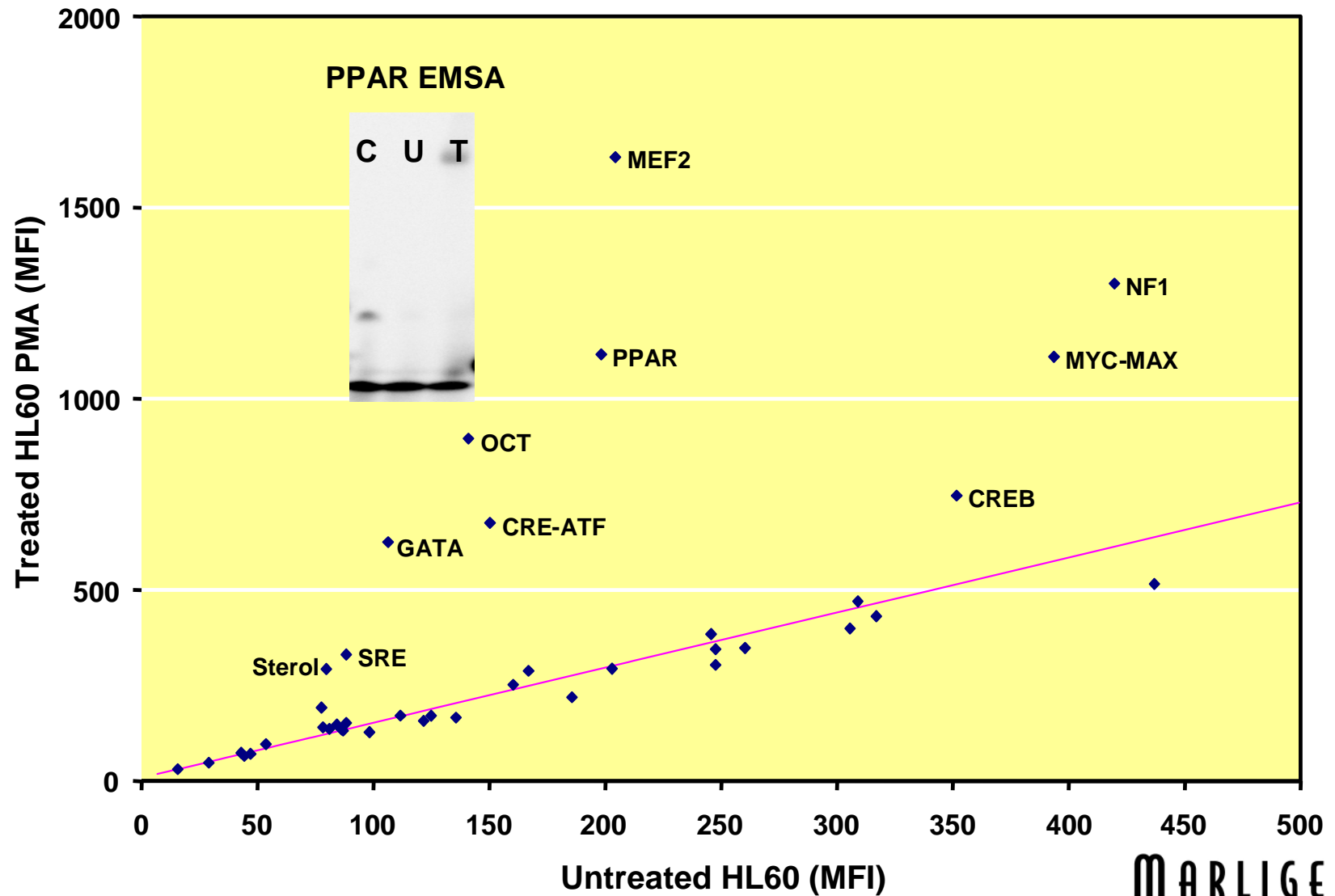
Transfections sometimes lead to unexpected results.



Treatment of HL-60 Cells with PMA/IM

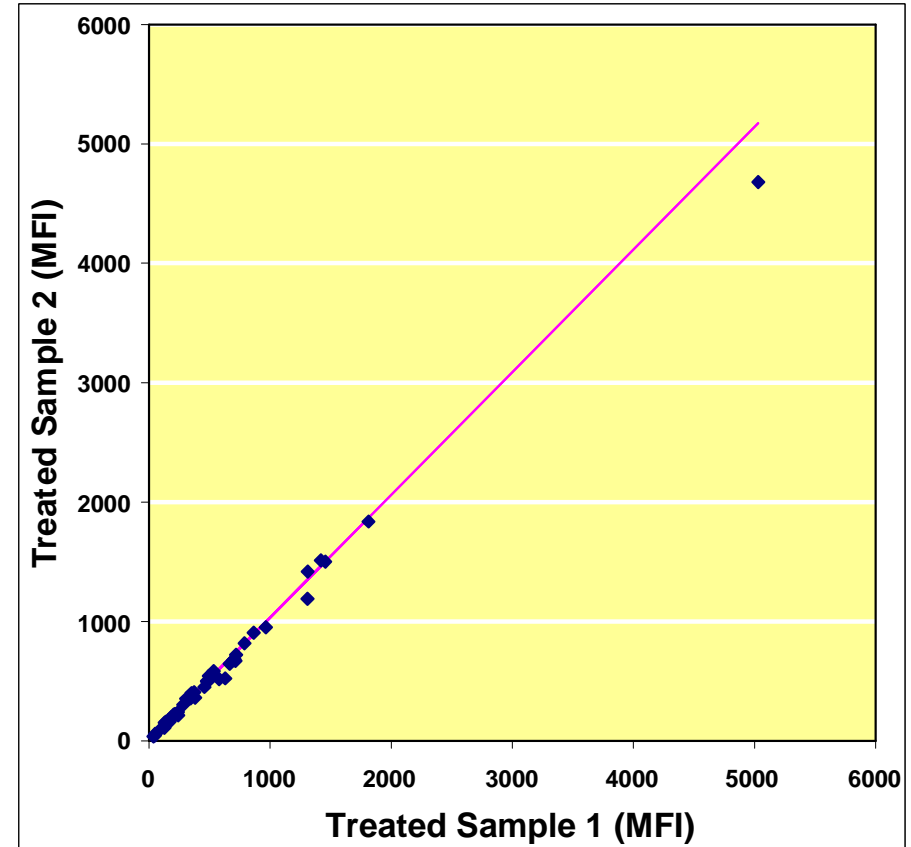
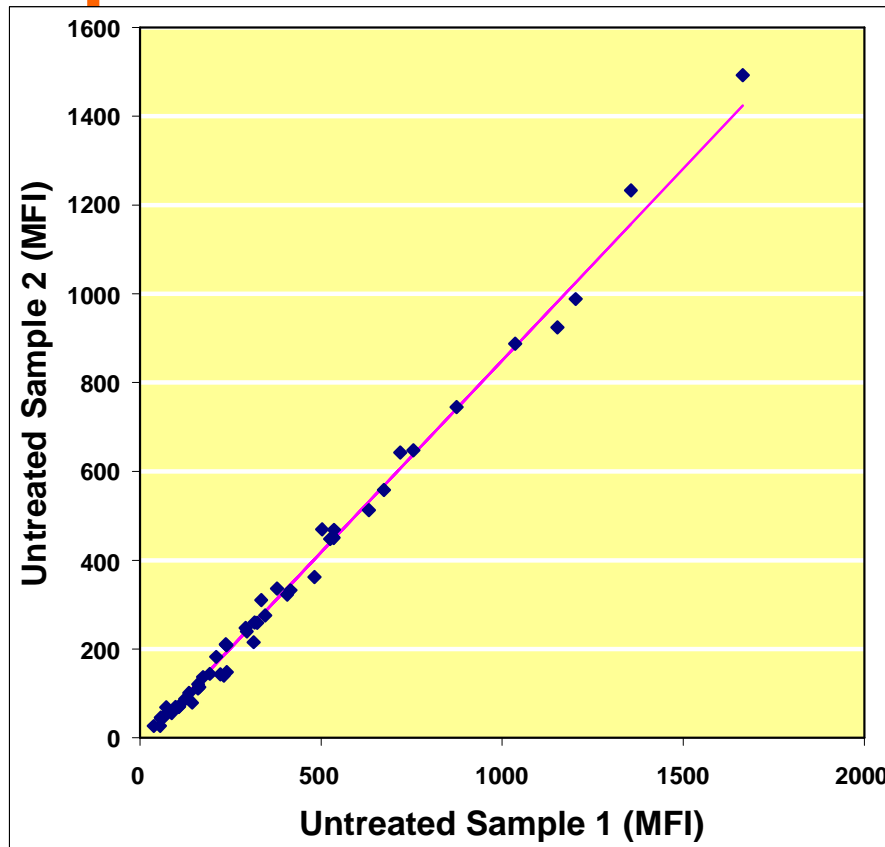


Treatment of HL-60 Cells with PMA/IM

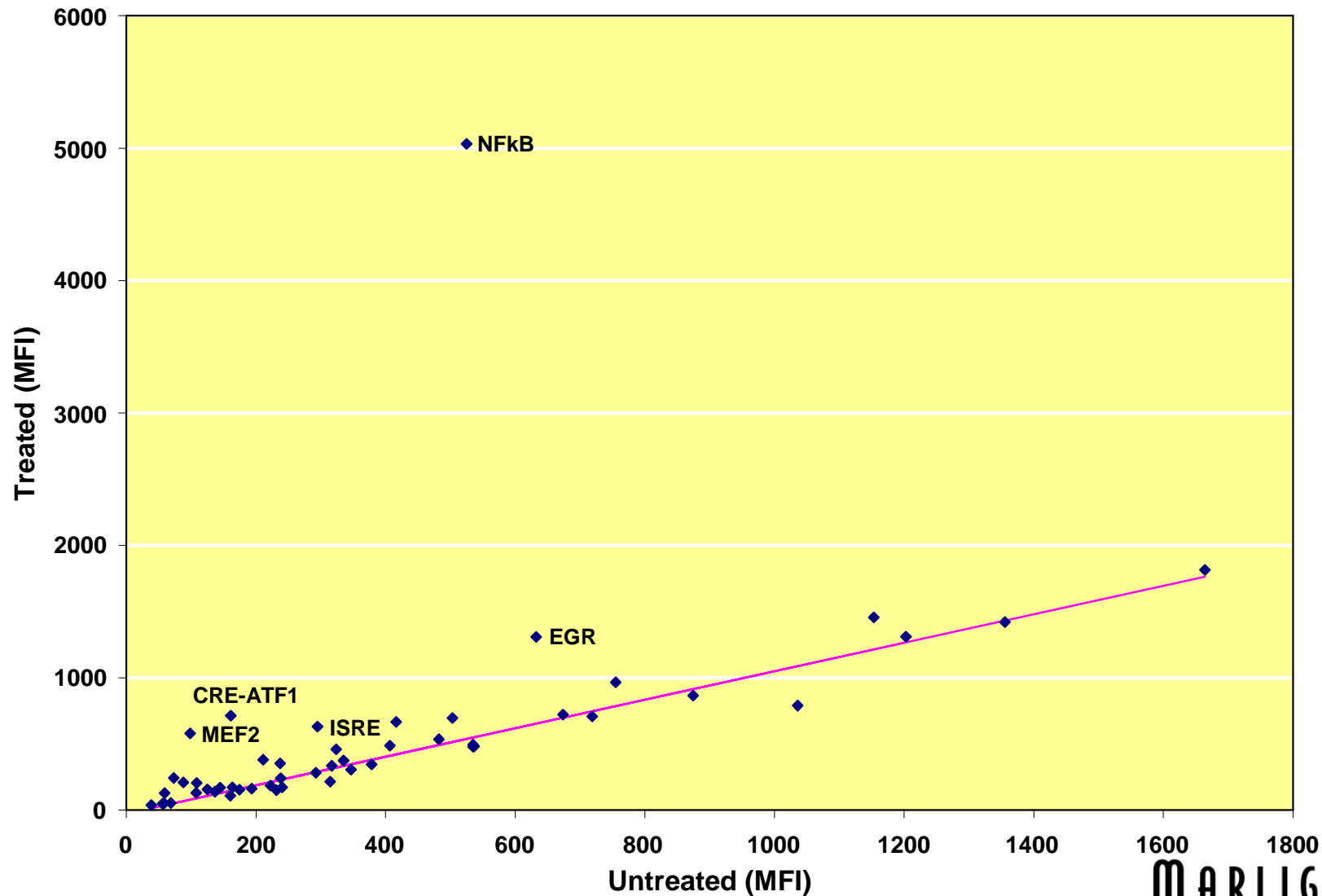


Whole blood treated with PMA/IM – 48 hrs

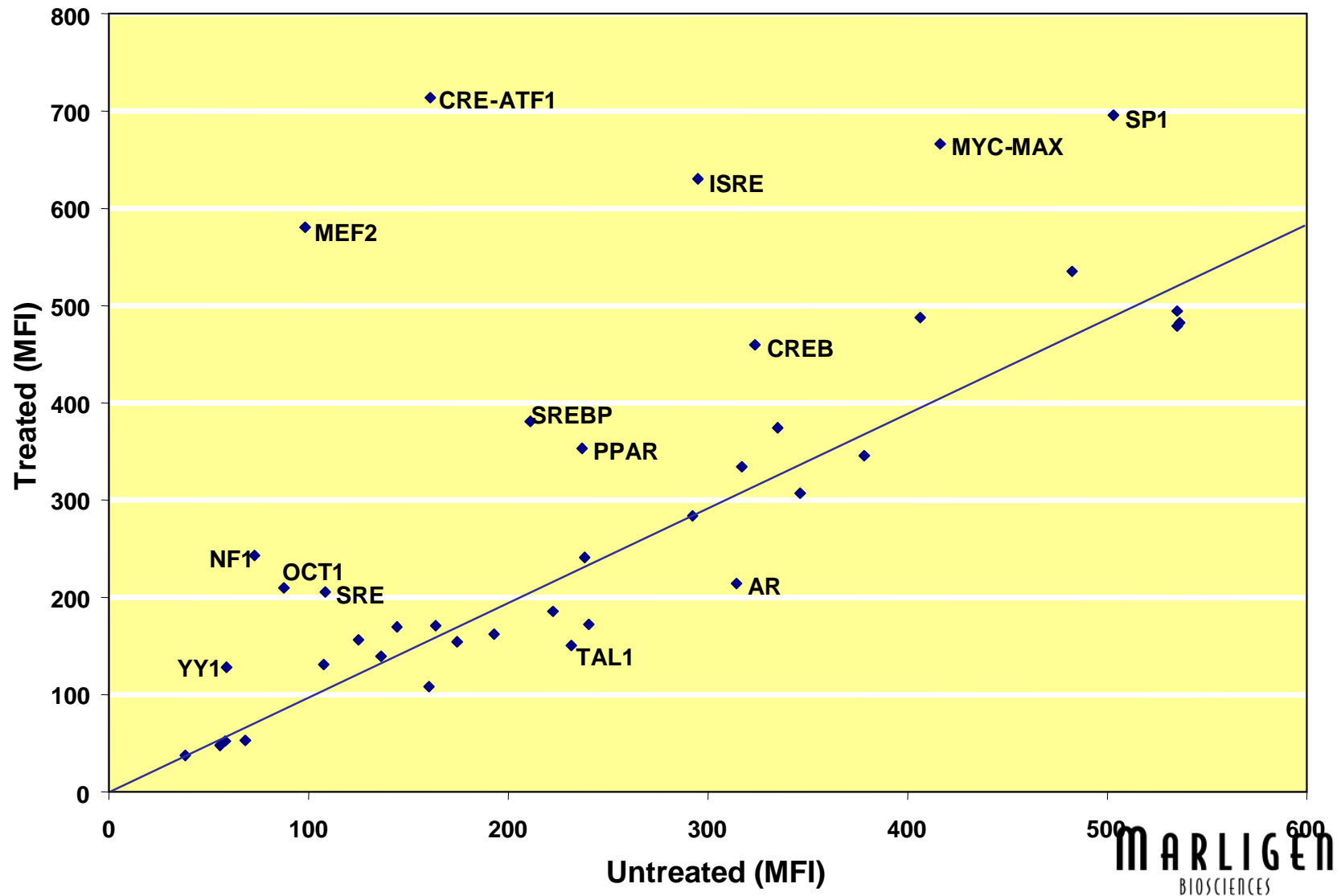
Sample to Sample Comparison



Whole blood treated with PMA/IM – 48 hrs



Whole blood treated with PMA/IM for 48 hrs



Similarities between PMA-treated HL-60 and Whole Blood

Assay	HL-60 PMA 24 hrs	Whole Blood PMA 48 hrs
EGR	7.10	2.0
MEF2	6.15	7.3
Octamer	4.81	3.1
GATA	4.37	1.2
PPAR	4.33	1.5
CRE-ATF	3.41	4.9
NFkb	2.86	9.3
SRE	2.75	2.3
Sterol	2.67	1.1
NF1	2.43	4.7
Myc-Max	2.21	1.6
HSF1	1.80	1.5
CREB	1.66	1.4
ISRE	1.32	2.2

Remaining Sites

	HL-60 PMA 24 hrs	Whole Blood PMA 48 hrs*
Min	0.9	0.7
Max	1.3	1.5
Median	1.05	1.0
Mean	1.07	1.2

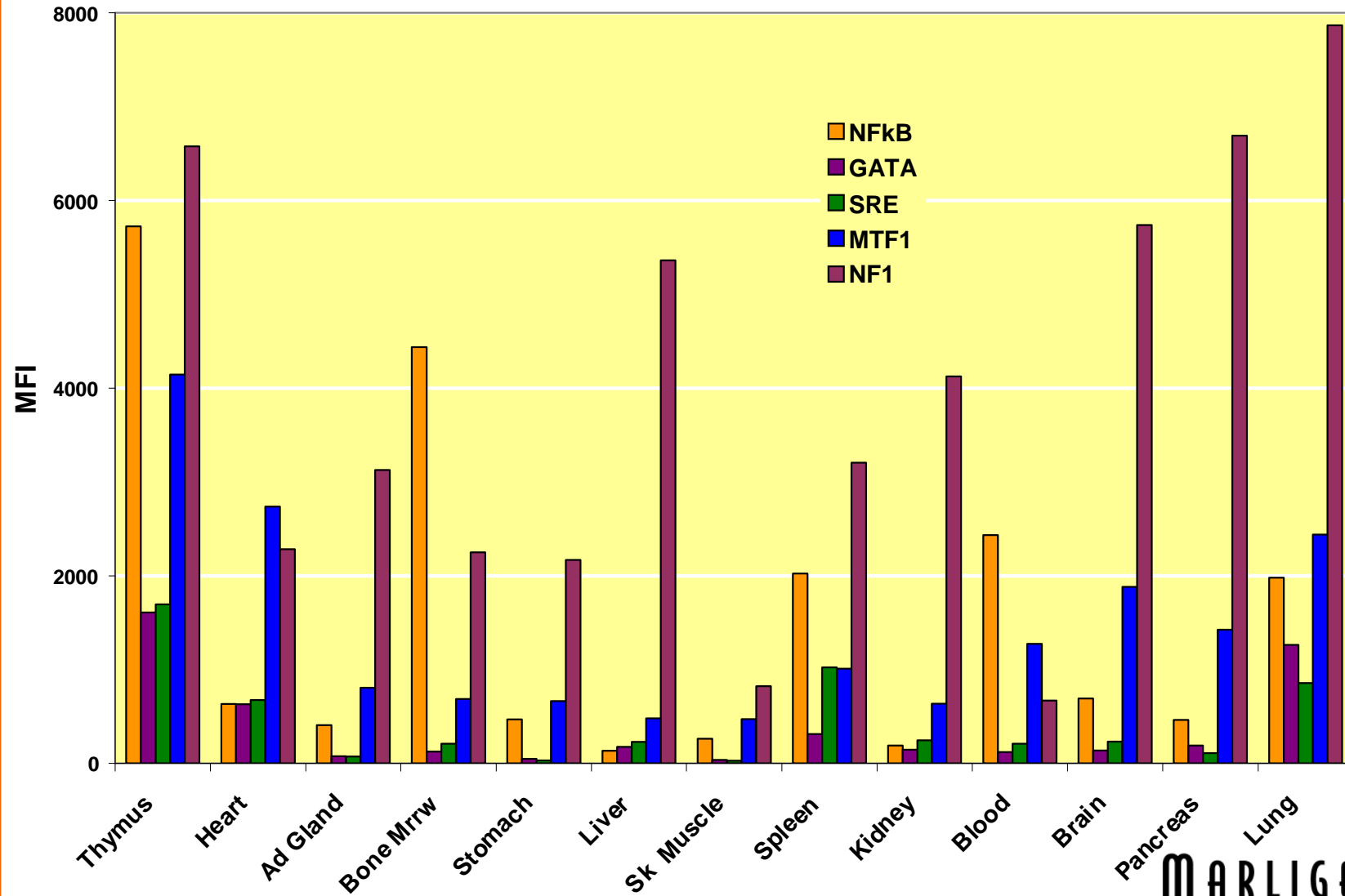
* Three sites with low signals excluded

Transcription Factor Profiling in Various Cell Lines

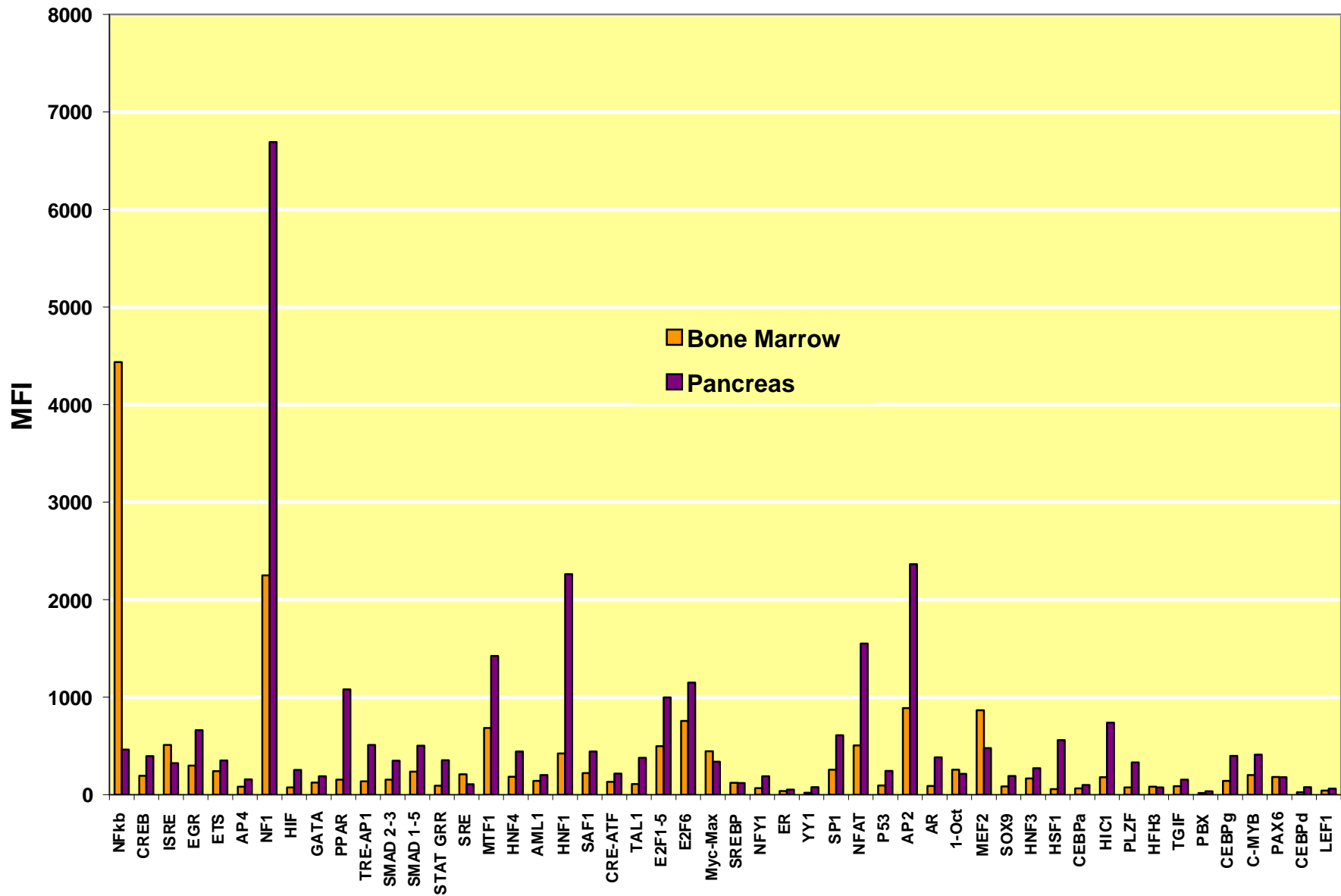
DNA binding activity has been measured in nuclear extracts from many different cell lines

Species	Cell Line	Cell Type
Human	293	Embryonic kidney
Human	RAJI	B-cell lymphoma
Human	MCF-7	Breast cancer epithelial
Human	HeLa	Epithelial from cervical carcinoma
Human	K562	Erythroleukemia
Human	HepG2	Hepatocellular carcinoma
Human	WI-38	Lung fibroblast
Human	U937	Monocytic leukemia
Human	KAS	Myeloma
Human	THP-1	Myelomonocytic leukemia
Human	U2OS	Osteosarcoma Epithelial
Human	HL-60	Promyelocytic leukemia
Human	Jurkat	T-cell leukemia
Human	MSC34	Mesenchymal stem cells (CD34+)
Mink	MV-1	Lung epithelial
Monkey	COS-7	Kidney
Mouse	C2C12	Myoblast
Mouse	NIH-3T3	Fibroblast
Rat	PC12	Phaeochromocytoma (neuronal)

Mouse Tissue Survey



Tissue Comparison across the 50-plex



Preparation Of Sample Is Very Important

- Assay sensitivity:
 - DTT must be > 0.2 mM
 - EDTA or EGTA must be < 1 mM
- For highest performance use Marligen's nuclear extraction kit buffers and protocol
 - Gradual increase in salt concentration to prevent loss of binding activity
 - Low spin cycles to prevent nucleus from being compacted and trapping transcription factors

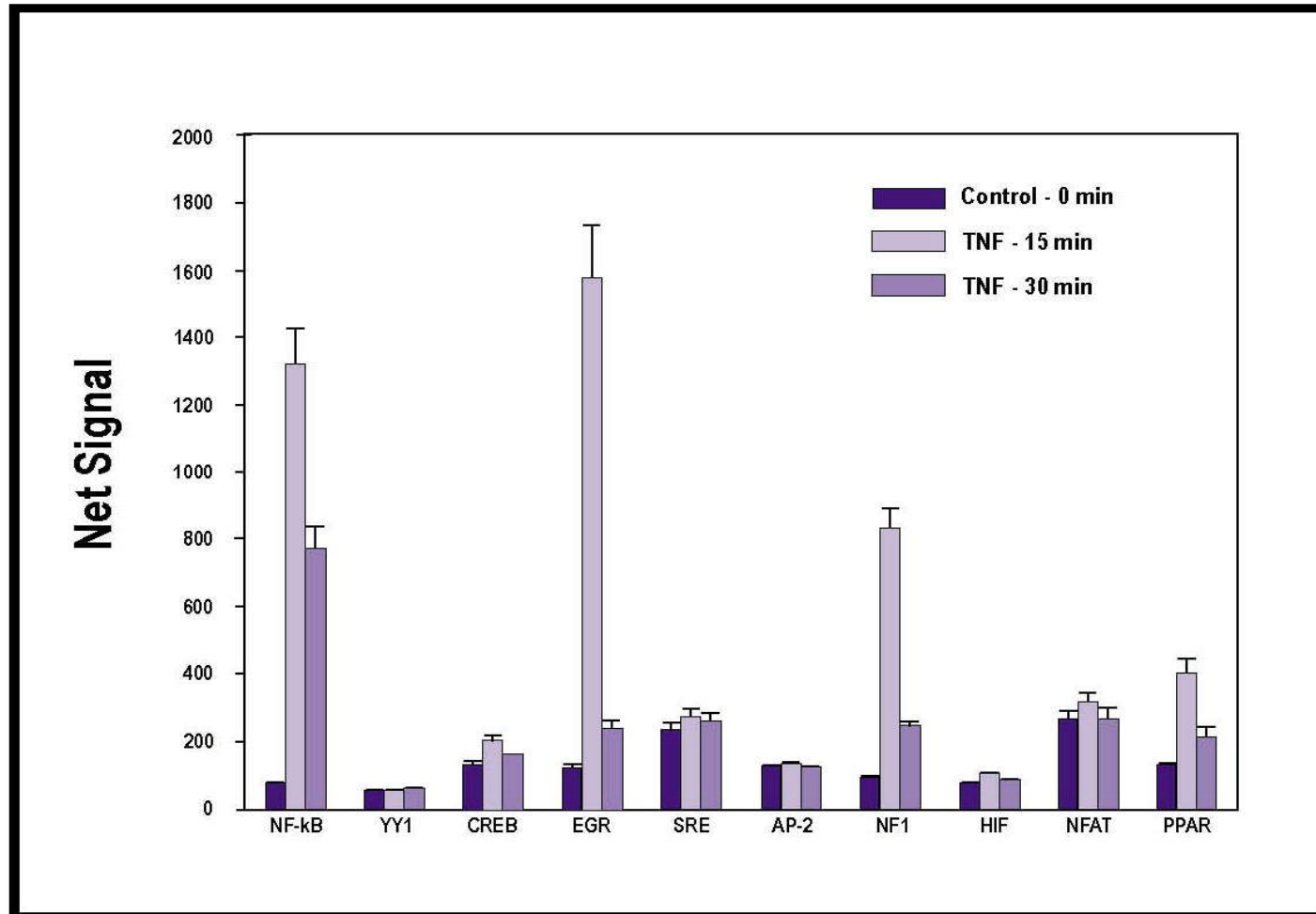


Studies and Applications



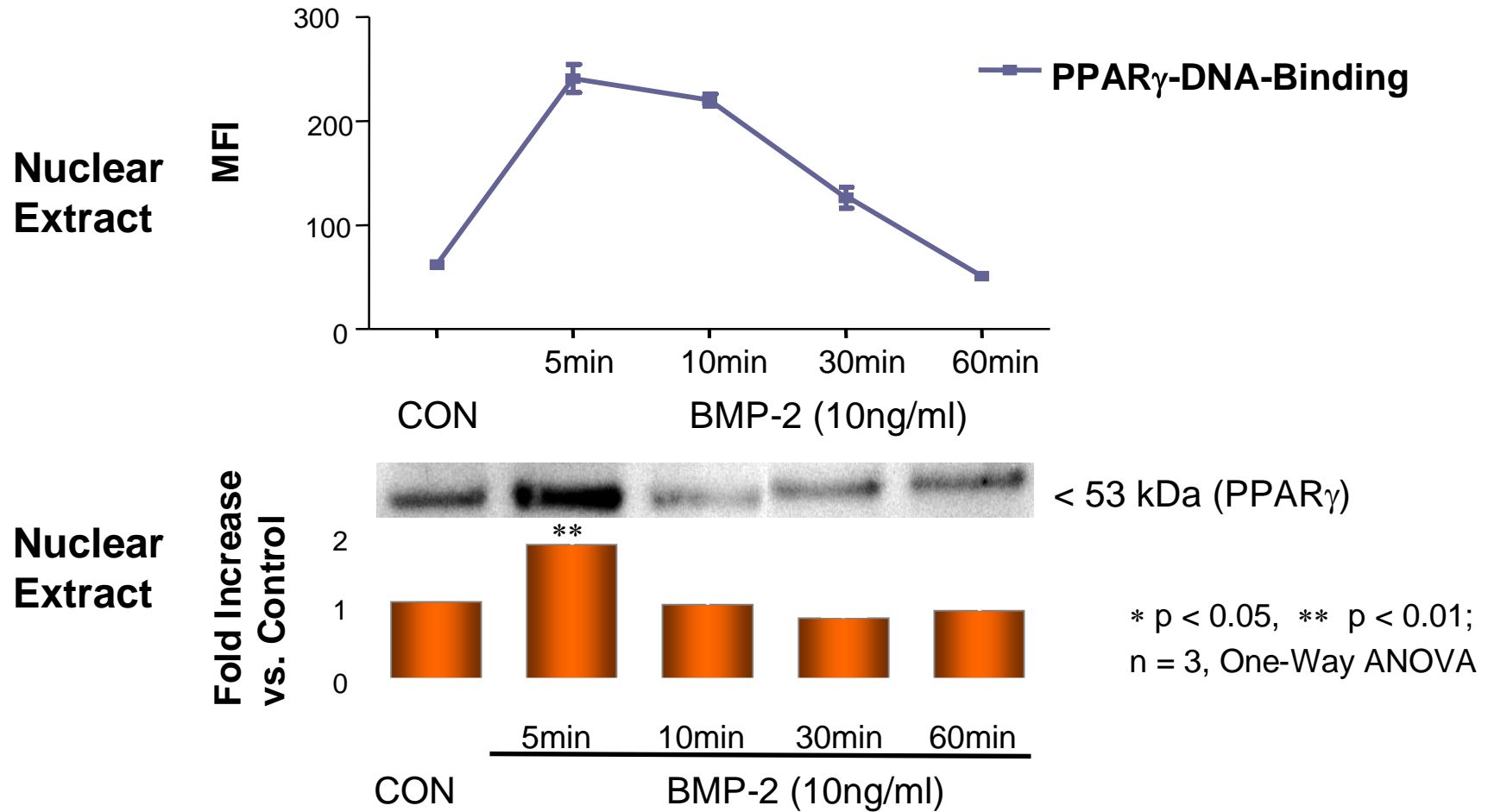
Novel Biological Effects Are Revealed Using the TF Multiplex Assay

Profile of transcription factor activation in dendritic cells stimulated with TNF- α



Data kindly provided by Anna Lokshin, University of Pittsburgh

Multiplex Assay Identifies Activation of PPAR- γ in SMCs stimulated with BMP-2



Hansmann G, Rabinovitch M (2005) *Circulation* 112 (17): II-154

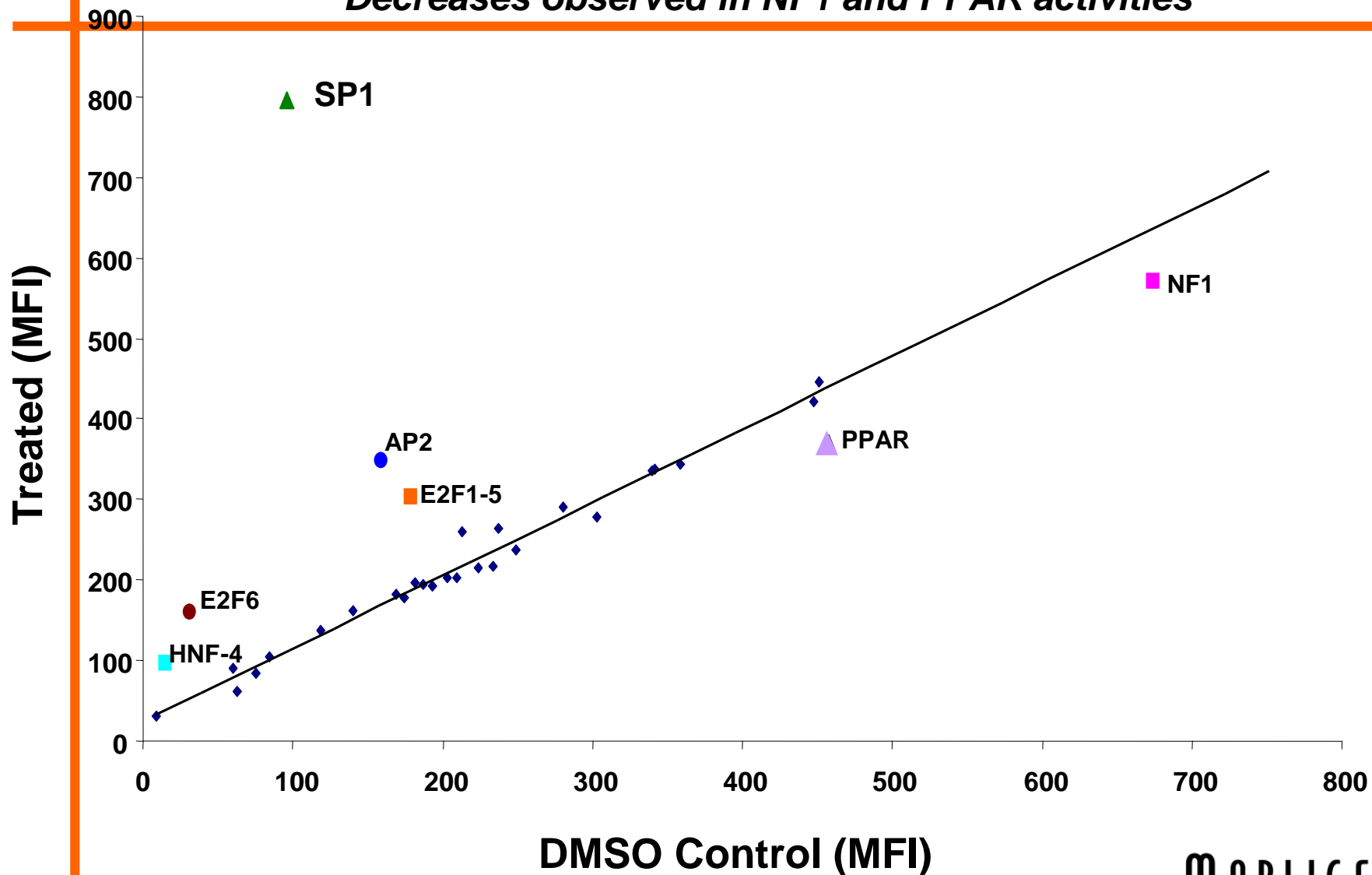
Use of Multiplex Assay in Drug Screening Applications

- Screening of compound libraries:
 - Screened 48 chemical compounds selected from a small chemical diversity library
 - Nuclear extracts were taken from Thp1 cells treated overnight in the presence of the compound (1 and 10 μM)
- Samples were evaluated using a 28-plex transcription factor assay.



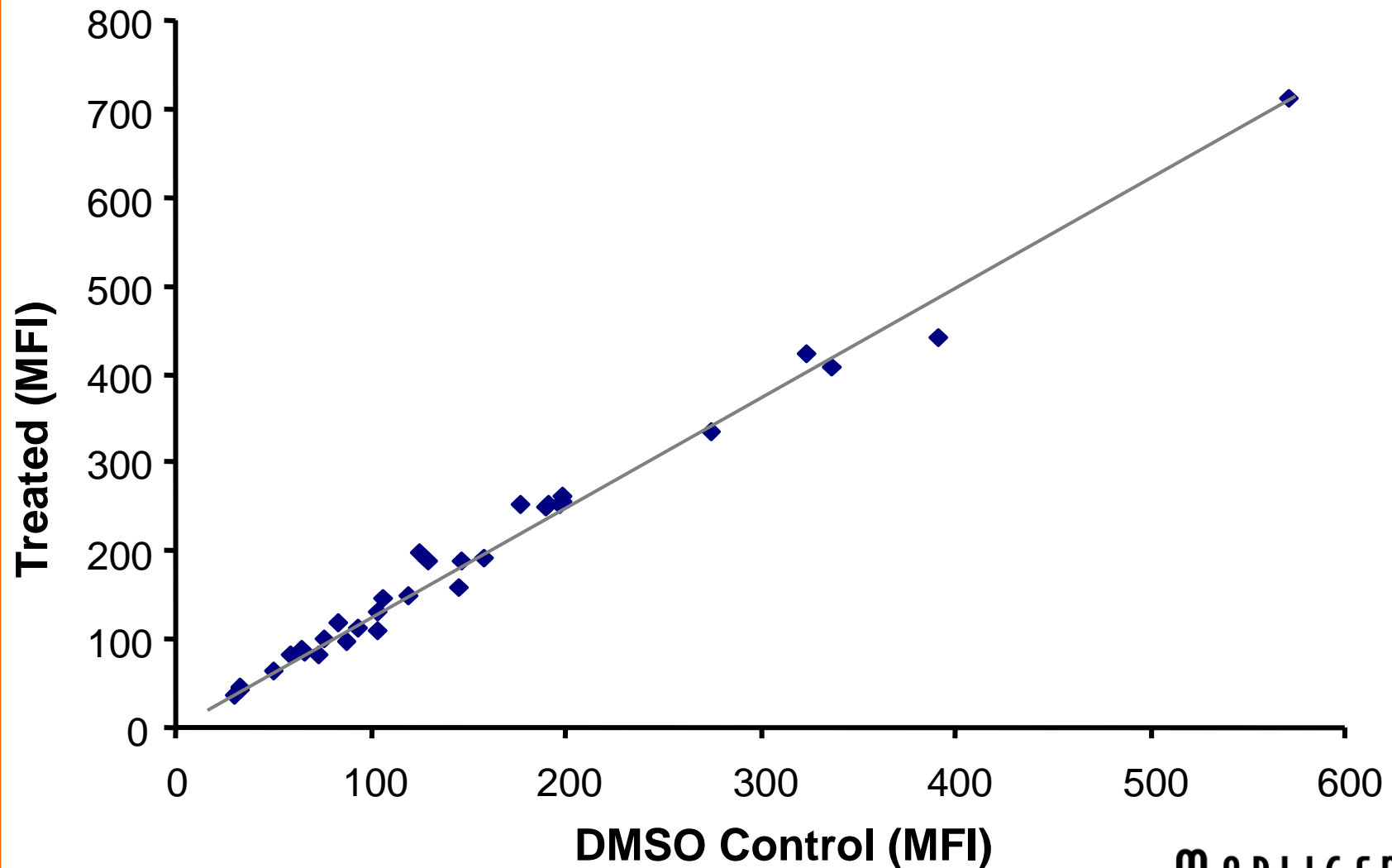
Multiplex Assay in Drug Screening

Compound #1 : Increases in AP2, E2F family, HNF4 and SP1 activities
Decreases observed in NF1 and PPAR activities



Multiplex Assay in Drug Screening

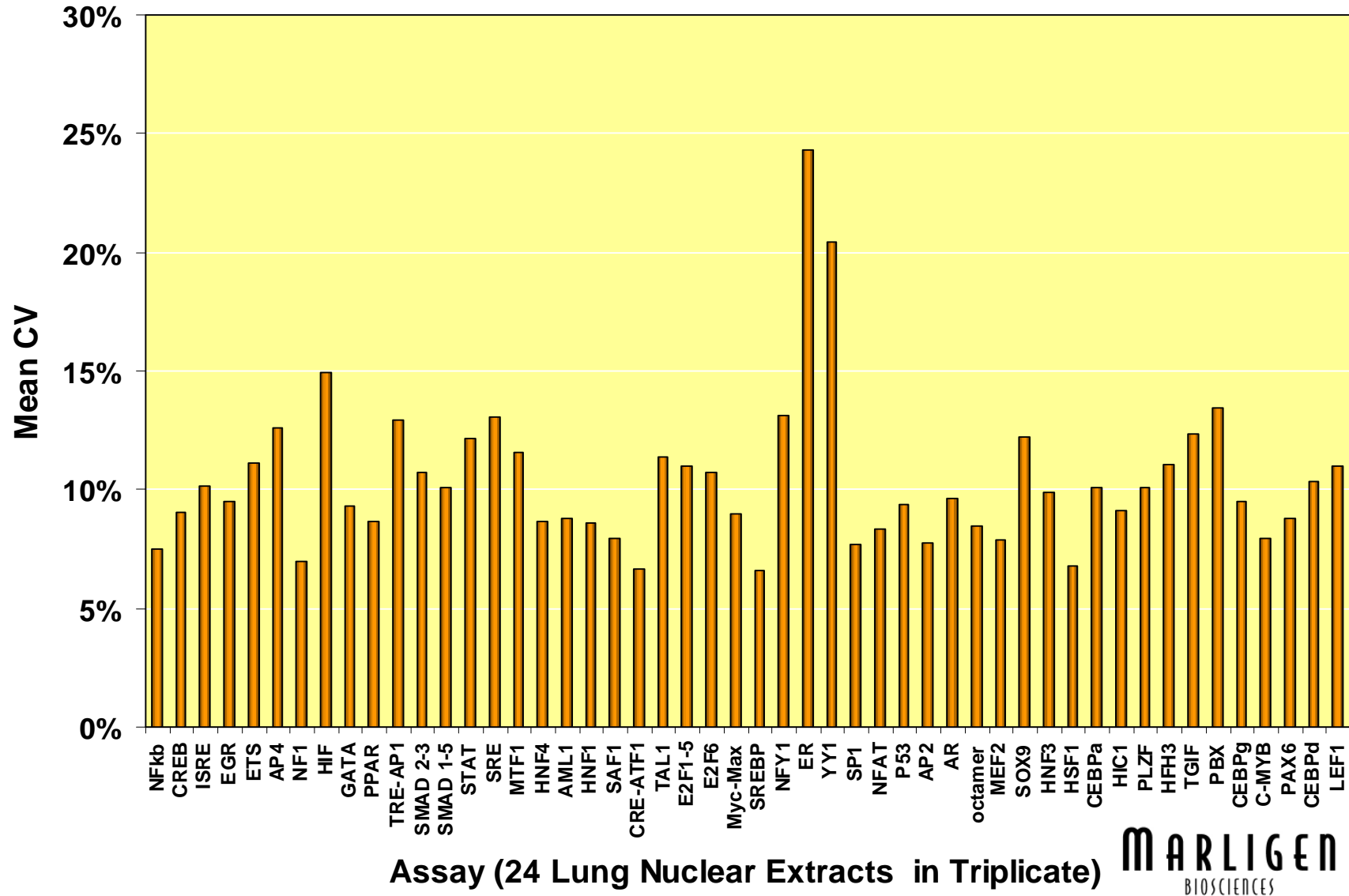
Compound #2 : No Significant Changes Observed



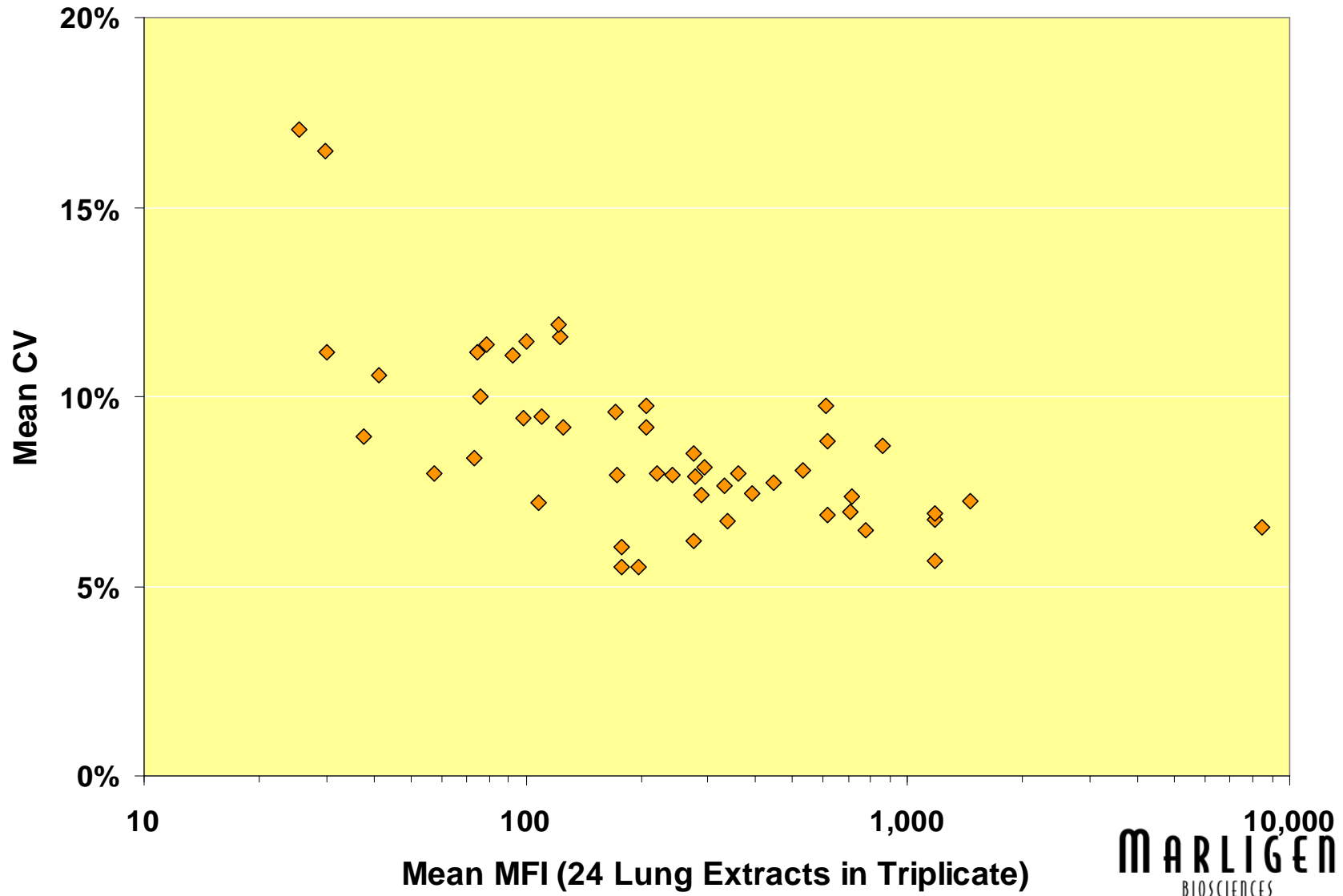
Drug Repositioning

- Major Pharma Study
- Six Groups of 4 mice
 - One group of control mice
 - Five groups of treated mice
- Lungs were removed and frozen
- Nuclear extracts were prepared from 100 mg lung tissue
- Each extract was assayed in triplicate with 10 μg of nuclear extract

Intra-Assay Reproducibility



Intra-Assay Variation

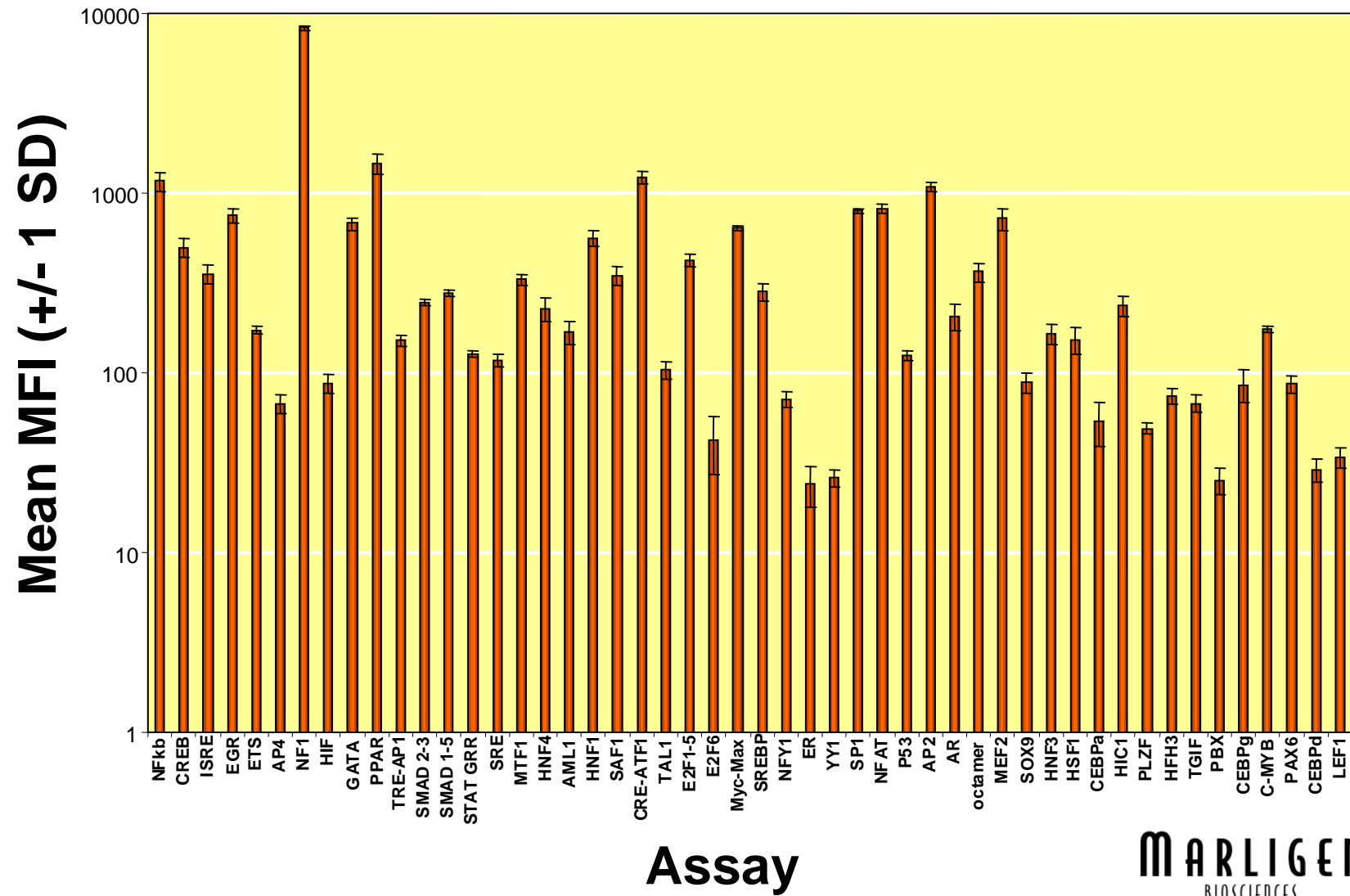


Inter-mouse reproducibility

Mouse Grp (N=4)	CV of the mean MFI	CV of the median MFI
A	20%	19%
B	21%	19%
C	15%	15%
D	18%	17%
E	11%	11%
F	20%	16%



Inter-mouse Variation



Mouse Model of Nickel Sulfate Exposure

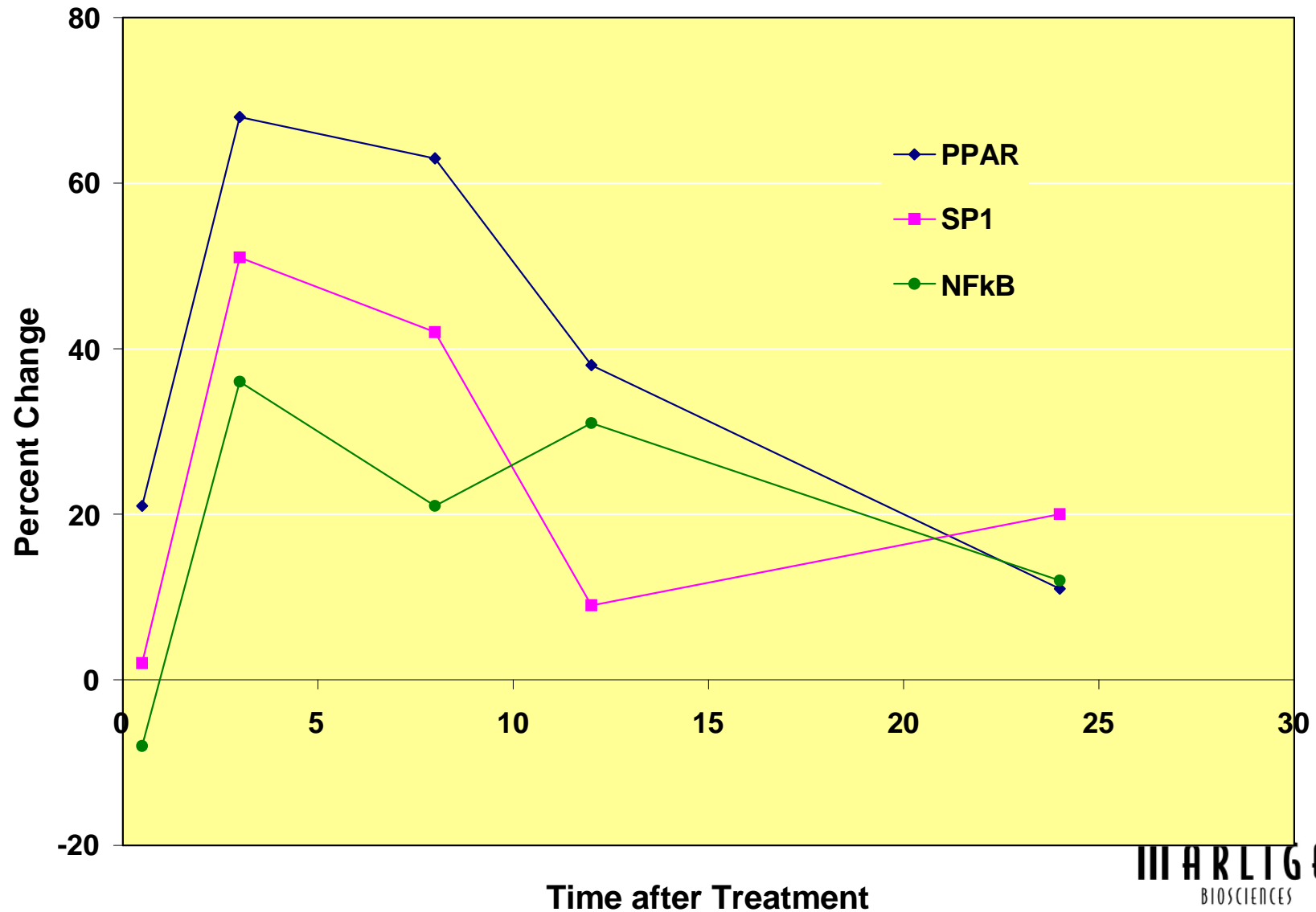
- Ambient and occupational exposures causes acute lung injury
- Mouse lung epithelial cells (MLE-15)
Treated with 600 μM nickel sulfate for 30 min, 3 h, 8 h, 12 h, and 24 h.
- Gene expression measured on 32K oligo array
- Transcription factor activity measured with 34-plex

Enriched Transcription Factors in Microarray Dataset

- 1129 significantly increased transcripts (FDR \leq 0.05).
- 802 significantly decreased transcripts (FDR \leq 0.05).

Transcription Factor	p Value
E2F1	0.001
AP-2	0.002
NF- κ B	0.003
Sp1	0.004
PPAR	0.009
HNF-4	0.009

Mouse Lung Time Course after NiSO₄



Biological/Physiological Significance to Nickel-Induced ALI

- **NF-kB:**
 - Regulates a number of genes relevant to ALI pathology (e.g., inflammatory chemokines, cell adhesion molecules, TNF-a).
- **Sp1:**
 - Has a role in regulation of surfactant gene expression, which is unequivocally necessary for normal pulmonary function.
 - Also regulates sodium channel, nonvoltage-gated genes that are important in fluid secretion and absorption across the lung epithelium.
- **PPAR:**
 - Regulates the expression of genes involved in lipid metabolism and inflammatory and reparative responses.

Significance of TF Profiling to Understanding Nickel-Induced ALI

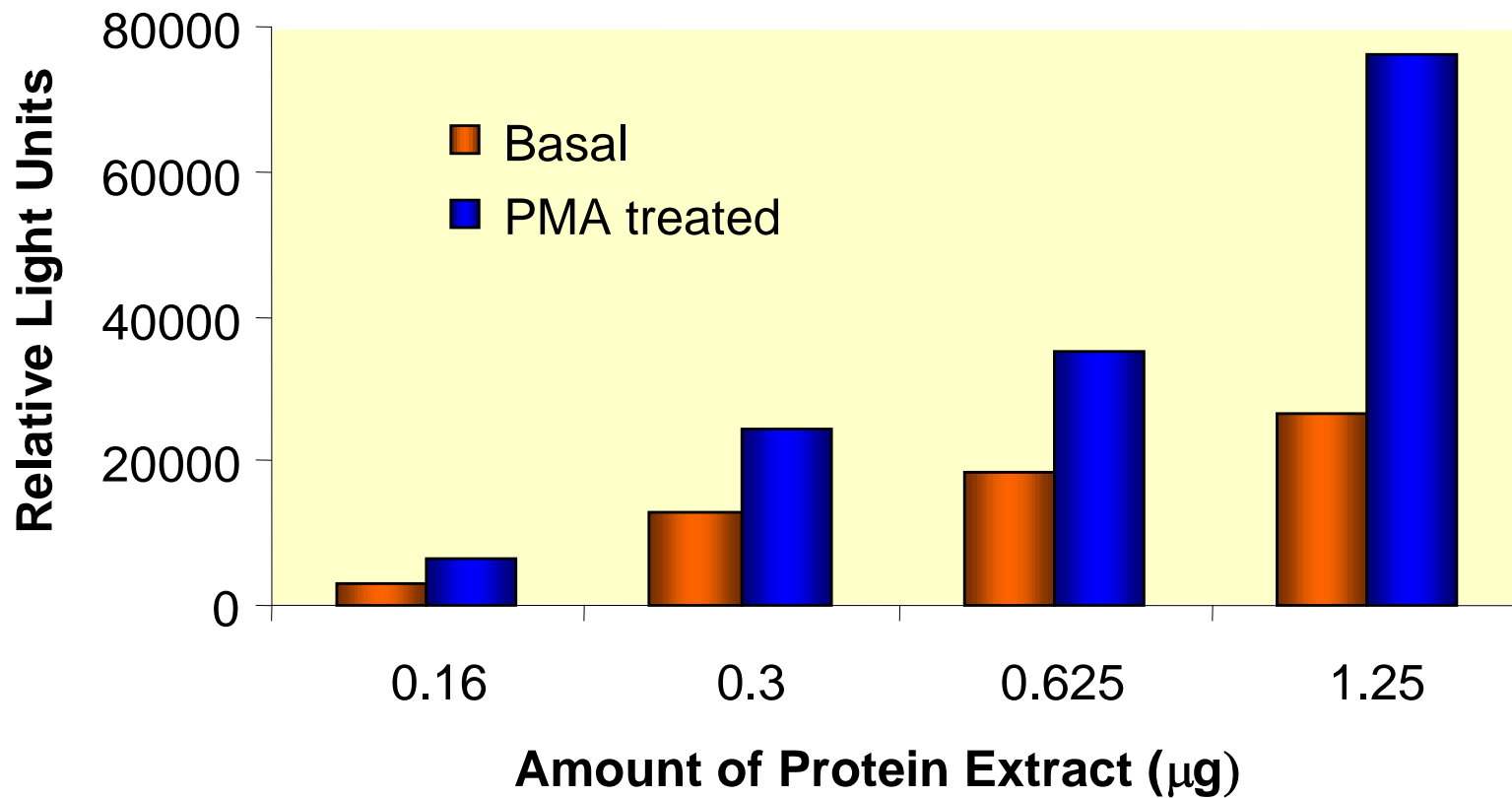
- TF and microarray assays allows for the identification and validation of overrepresented transcription factors involved in a given cellular response (e.g., lung epithelial cells to nickel),
- Determination of known and novel differentially expressed genes that may be regulated in part by these transcription factors.

Comparisons to EMSA and ELISA

	EMSA	ELISA	Marligen plate	Multiplex
Detection	radioactivity	colorimetric	chemi-luminescent	fluorescent
Time to result	1-2 days	4 hours	4 hours	3-4 hours
Quantitative	No	Yes	Yes	Yes
Min. detectable sample	~20 µg sample	~3.5 µg sample	< 0.25 µg sample	~ 0.5 µg sample
Result/sample	1	1	1	Up to 50
Max. results for 1µg	0	0	4	40
5µg	0	1	20	200
20µg	1	5	80	800
Cost/result 1 plex	\$3.00-10.00	\$6.00	\$5.46	\$5.18
3 plex	-	-	-	\$3.52
10 plex	-	-	-	\$2.95
20 plex	-	-	-	\$1.50

Microplate Format can Detect TF Activity in Less Sample

PPAR activation was observed in as little as 0.16 μg of nuclear extract from PMA-treated Thp-1 cells



Acknowledgements

- Marligen
 - Pete Clausen
 - Fiona Coats
 - Imani Jones
 - Mike Connolly
 - Alec Patrick
- University of Cincinnati
 - Scott C. Wesselkamper
 - George D. Leikauf
 - Maureen A. Sartor
 - Xiangdong Liu
 - Mario Medvedovic
 - Craig R. Tomlinson
- University of Pittsburg
 - Anna Lokshin
 - Brian Nolan
- Stanford
 - George Hansmann
 - Maria Rabinovitch
- University of Tuebingen
 - Thomas Joos
 - Oliver Poetz

