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Proximity Ligation Assays, A Method For Ultra Sensitive Protein Detection

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Staff Scientist
Thermo Fisher Scientific

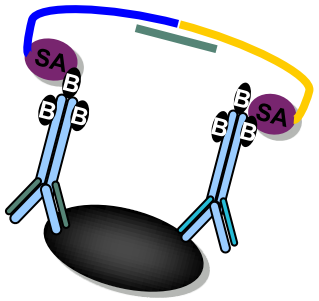
What Do Proteomic Tools Need To Do?

- Measure protein abundances
  - Protein concentrations can differ by more than 1000-fold
- Measure protein variations
  - Isoforms - the number of mature proteins is estimated to far exceed the number of genes
- Measure protein modifications and protein interactions
  - Can regulate the protein dynamics
    - Activity
    - Localization
    - Stability
- Measure proteins on a large scale
  - Entire pathways
  - Entire cellular systems

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PLA® Technology: A Perfect Union

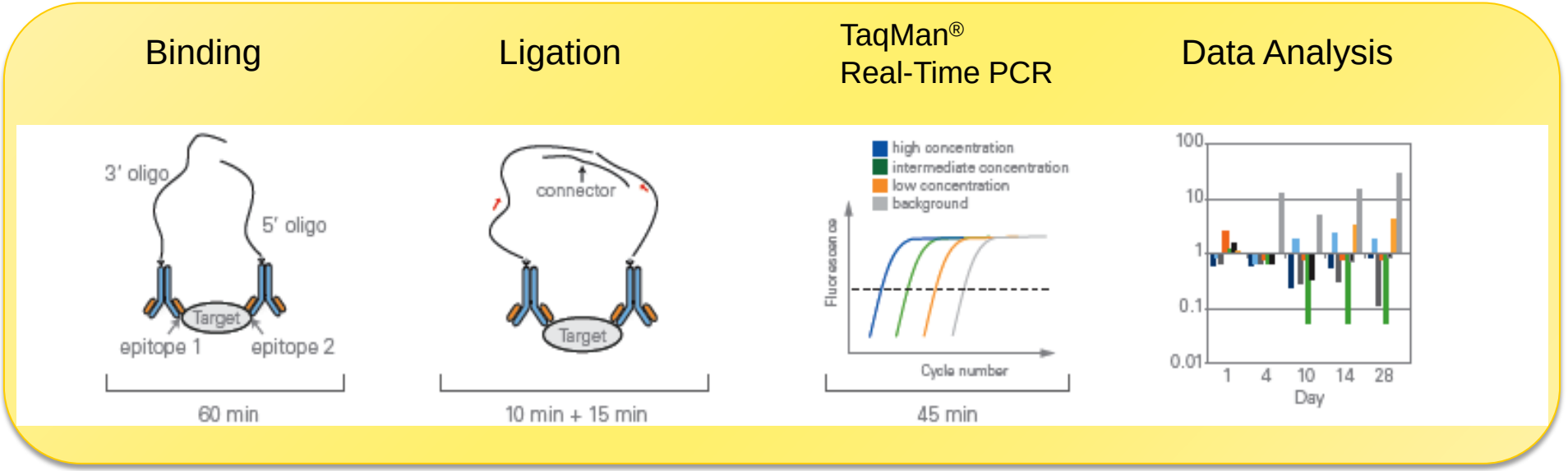
- PLA® combines the best of “two worlds”:
  - The simplicity, sensitivity, and broad assay dynamic range of qPCR
  - The specificity of affinity-based protein detection reagents
- PLA® is flexible, scalable (up and down)



- PLA® enables a multi-dimensional analysis of DNA, RNA, and proteins on a single detection platform

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TaqMan® Protein Assays (TPA) utilize PLA® Technology



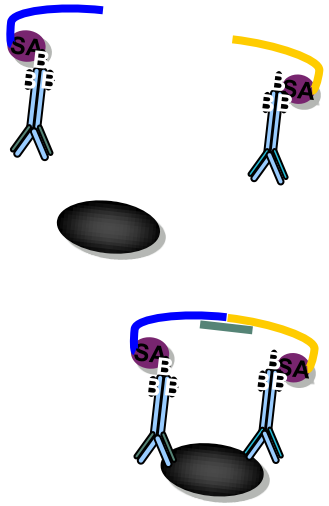
- Assay based on two antibodies, each conjugated to a different oligonucleotide (one 3' oligo, one 5' oligo)
- When the two conjugated antibodies bind and are in close proximity, the oligonucleotides can be ligated, serving as the template for real-time PCR amplification and quantification

Fredriksson, S., et al. *Nature Biotechnology* **20**, 473 - 477 (2002)

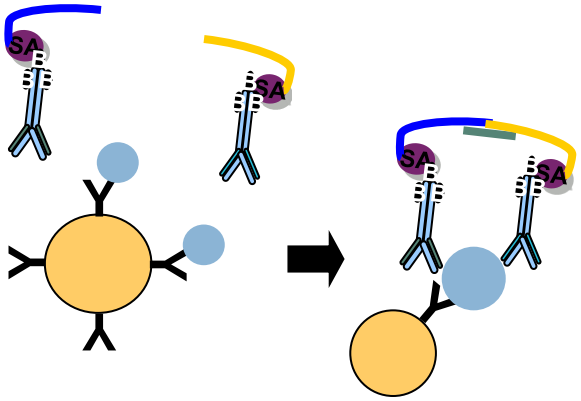
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PLA® Platform Options: Homogeneous or Solid-phase

- **Option 1: Homogeneous Assays**
  - Rapid (<3hrs TTR), simple (no washes)
  - Dynamic ranges of ~3-4+ logs
  - Ultra-sensitive (e.g. LOD <0.1pg/mL)
  - Low sample input (1-2µL)



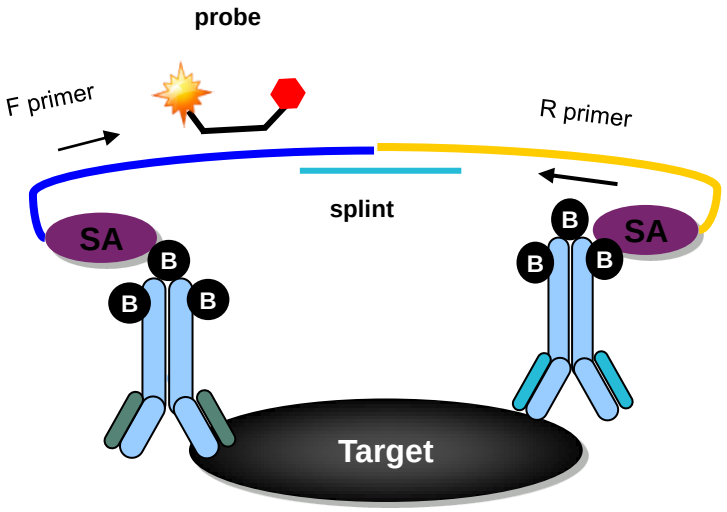
- **Option 2: Solid-phase Assays**
  - Ultra-sensitive (e.g. LOD <0.1pg/mL)
  - Broader dynamic ranges, 6+ logs
  - ~5hrs TTR
  - Plate or bead-based assays
  - Higher sample volume input (10-25µL)



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Design Elements: The Oligos

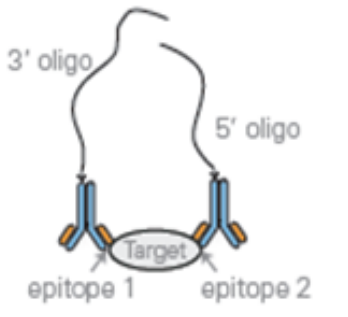
- Oligo sequence elements –
  - Oligo A: “left” oligo, ~60mer, 3’ OH
    - Forward primer site
    - Probe site
    - Splint overlap region
  - Oligo B: “right” oligo, ~40mer, 5’ P
    - Reverse primer site
    - Splint overlap region

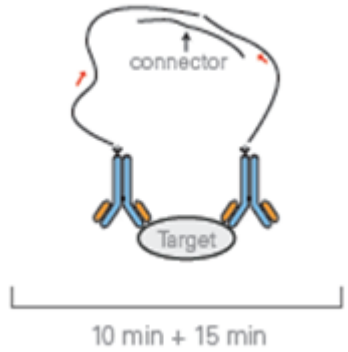


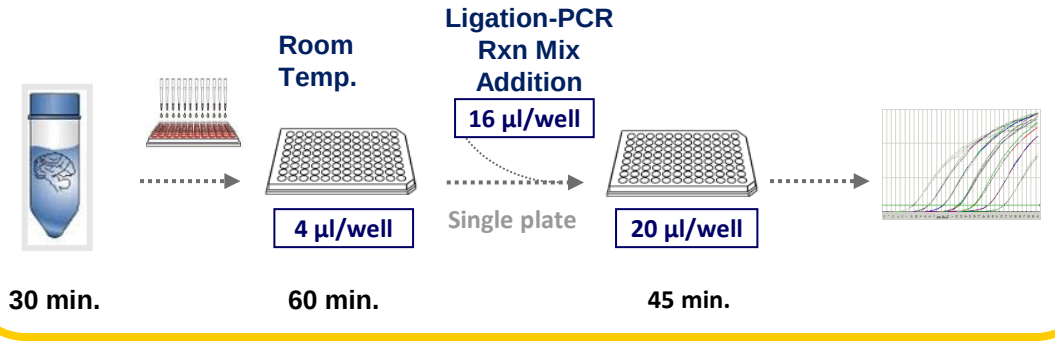
- Oligo conjugation
  - Indirect – “universal” oligo attachment to streptavidin (SA)
    - SA-oligo to be used in conjunction with biotinylated antibodies
    - Requires that antibodies are biotinylated

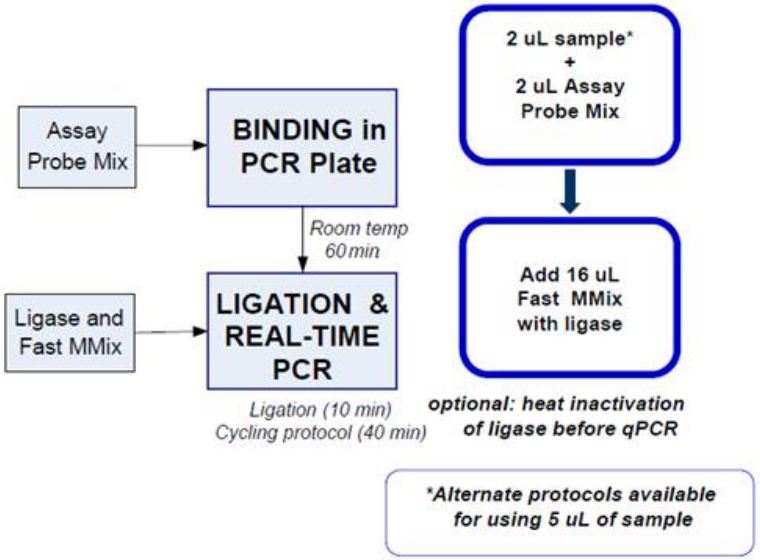
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Simple, Flexible Homogeneous Workflow: TPA II



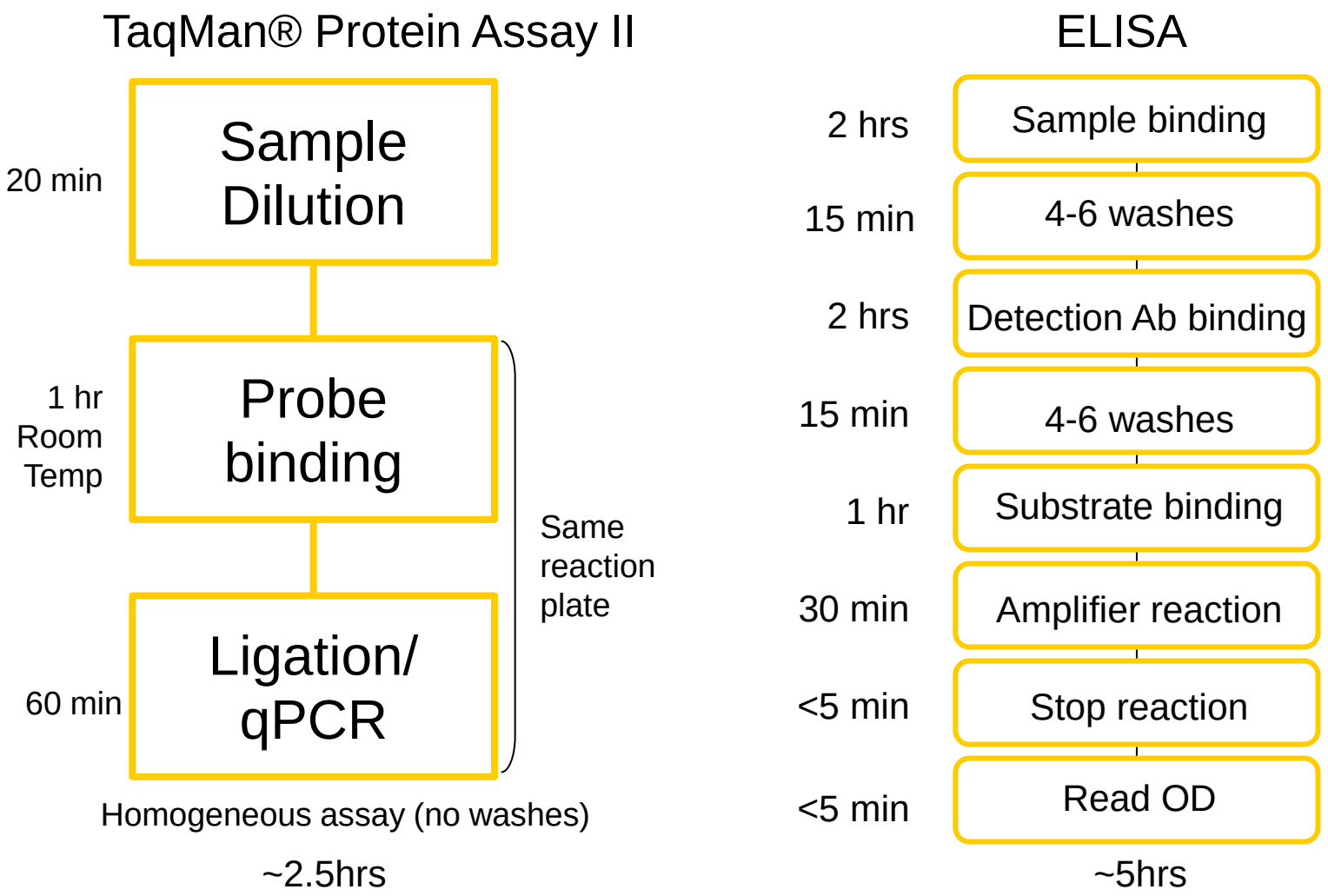






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Comparing Workflows: TPA II and ELISA

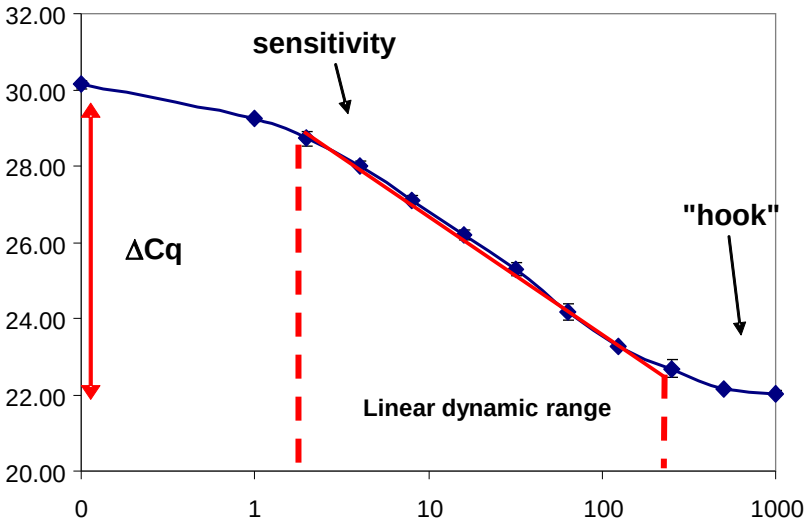


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Experimental Design Considerations: Running an Assay

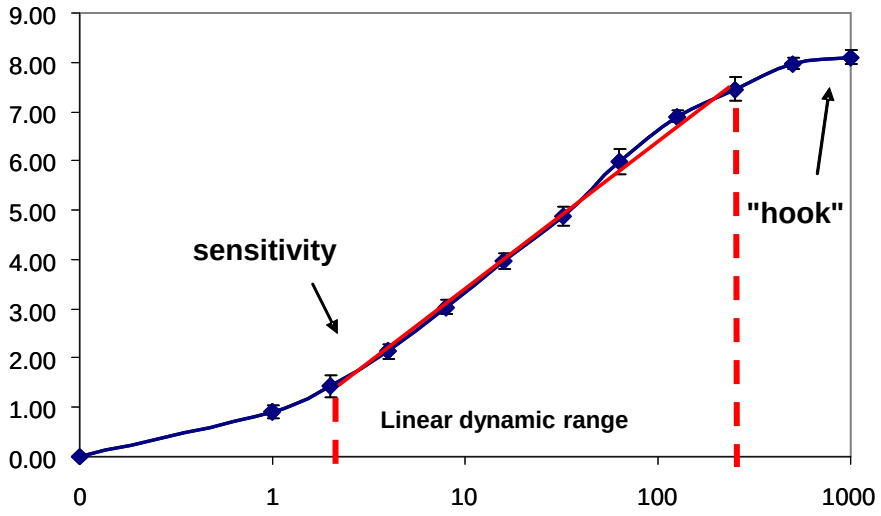
- Sample type – isolated protein, cells, tissues, body fluids (e.g. serum/plasma)
- Assay dynamic range and sample input
- Method for quantification – relative or ‘absolute’

CSTB Expression in Raji



C_q vs cell input

CSTB Expression in Raji

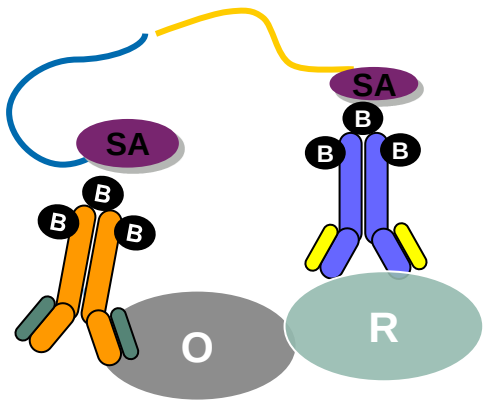


ΔC_q vs cell input

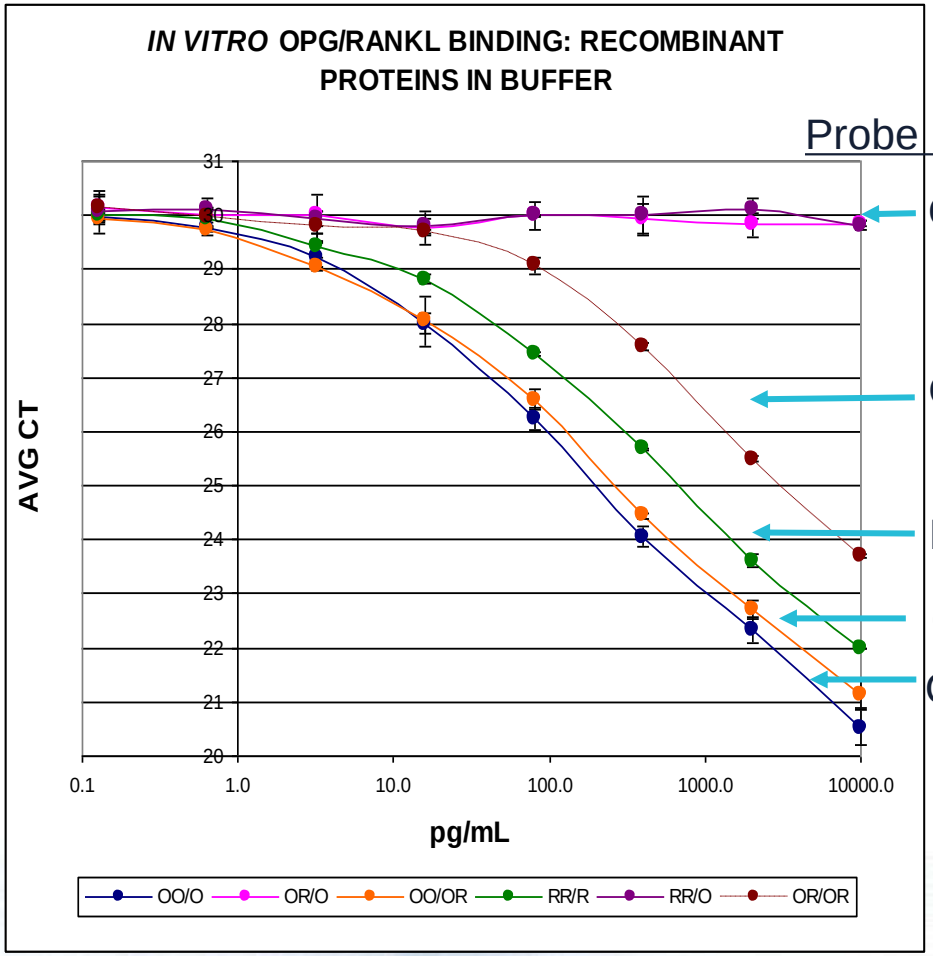
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In vitro interactions between OPG and its ligand (RANKL) demonstrate specificity of TPA probe binding

OPG (Osteoprotegerin) is a circulating 'decoy' receptor for RANKL (receptor activator of NF-kappaB ligand) in bone metabolism.



OPG RANKL



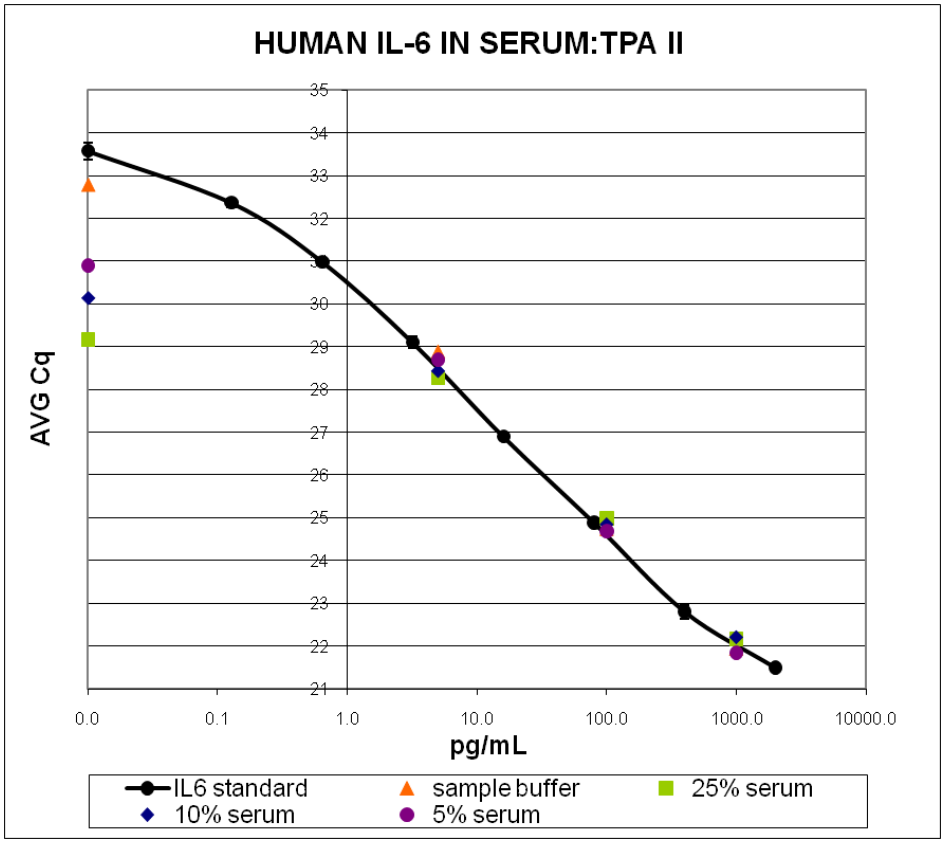
Specific interactions between OPG and RANKL can be detected

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Detection of Proteins in Serum Samples Can Be Extremely Sensitive (e.g. IL-6)

- Assay dynamic range
  - 0.128-2000pg/mL
- Sample = diluted serum
  - 4-fold, 10-fold, and 20-fold dilution in sample buffer

Sample Name	Raw Value Average	Raw Value Stdev	Conc. Expected	Conc. Average	% Recovery	Raw Value %CV	pg/mL	% recovery average
sample buffer	68832.2	2172.3	1000	1000.7	100.1	3.2		86.9
	9877.7	889.6	100	86.2	86.2	9.0		
	555.5	21.3	5	3.7	74.4	3.8		
	30.9	0.0	0	0.0	NA	0.0		
25% serum	58826.1	4644.7	1000	770.2	76.7	7.9		79.0
	8284.1	314.6	100	71.0	79.0	3.8		
	856.1	116.4	5	6.0	81.3	13.6		
	458.4	64.7		3.0	NA	14.1	12.0	
10% serum	57050.4	8183.8	1000	739.8	73.8	14.3		89.4
	9130.3	1057.1	100	79.0	90.2	11.6		
	755.6	28.3	5	5.2	104.2	3.7		
	233.6	36.3		1.4	NA	15.6	13.6	
5% serum	73146.8	5795.6	1000	1129.0	112.8	7.9		104.2
	10204.2	721.2	100	89.3	102.8	7.1		
	633.4	33.1	5	4.3	97.2	5.2		
	136.7	5.3		0.7	NA	3.9	13.9	



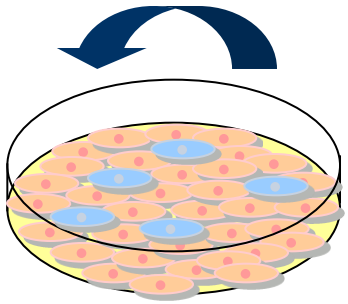
- Spike recovery in serum (5, 100, and 1000pg/mL)
- Acceptable range, 70-130%

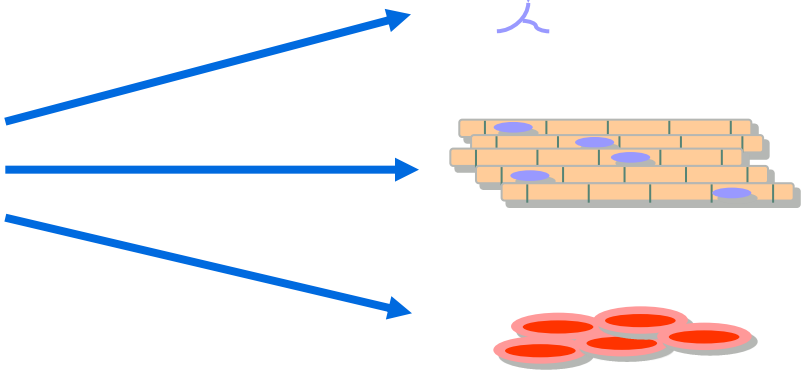
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Detection of Stem Cell Pluripotency Markers

Pluripotent cells: Self renewal → **Loss of Pluripotency:** Differentiation

Embryonic Stem Cells





Neural cells (neurons)

Mesenchymal cells (connective tissue)

Hematopoietic cells (blood)

Key Markers of Pluripotency

NANOG	OCT3/4
SOX2	LIN28

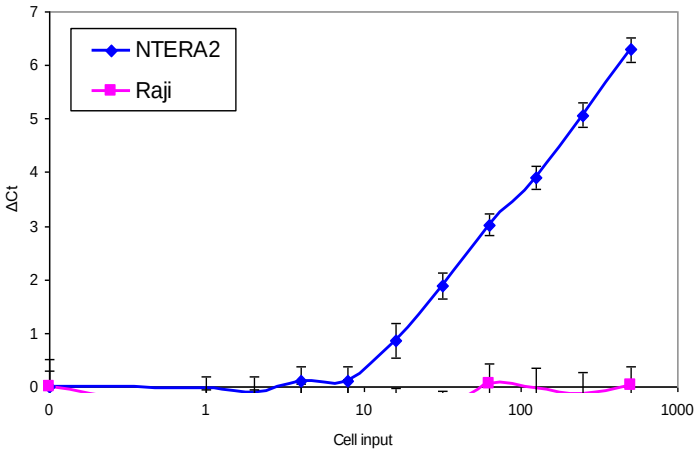
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TaqMan® Protein Expression Assays for Stem Cell Markers are Specific and Sensitive

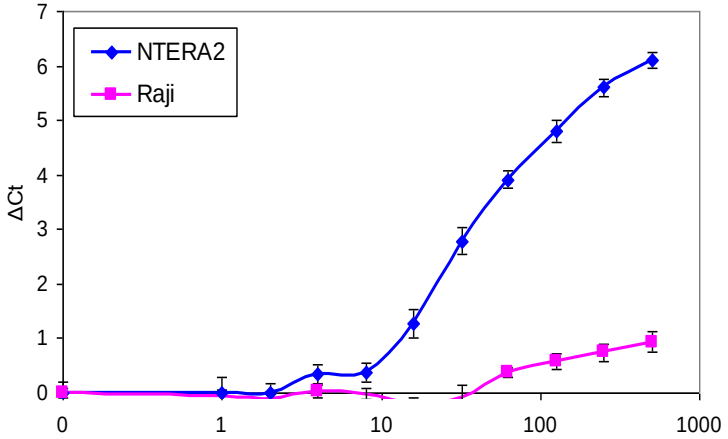
NTERA2 lysate – positive control

Raji lysate – negative control

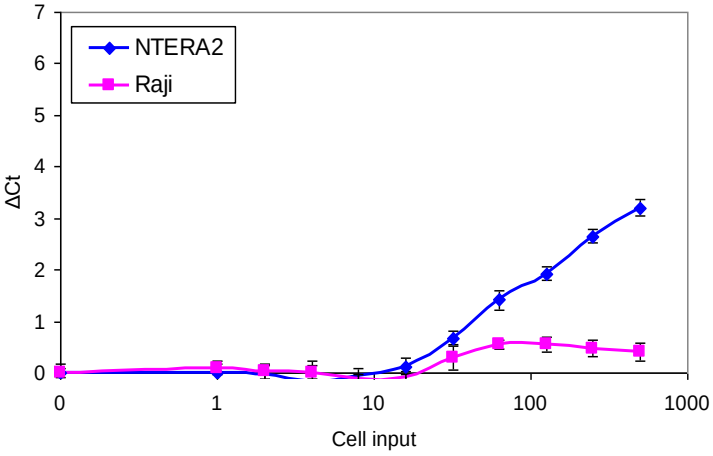
SOX2



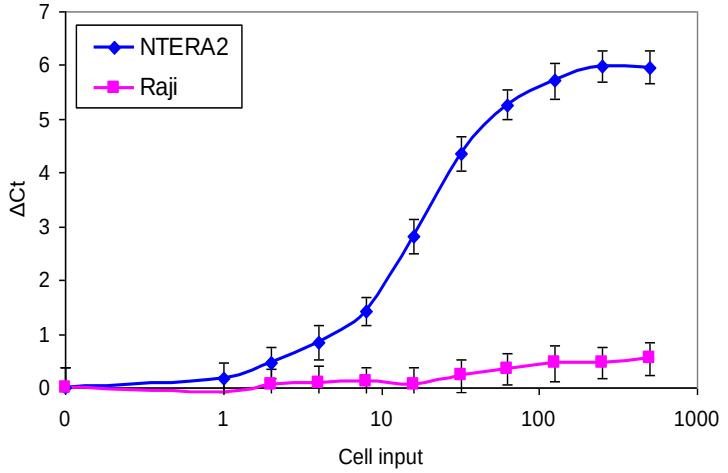
OCT3/4



NANOG

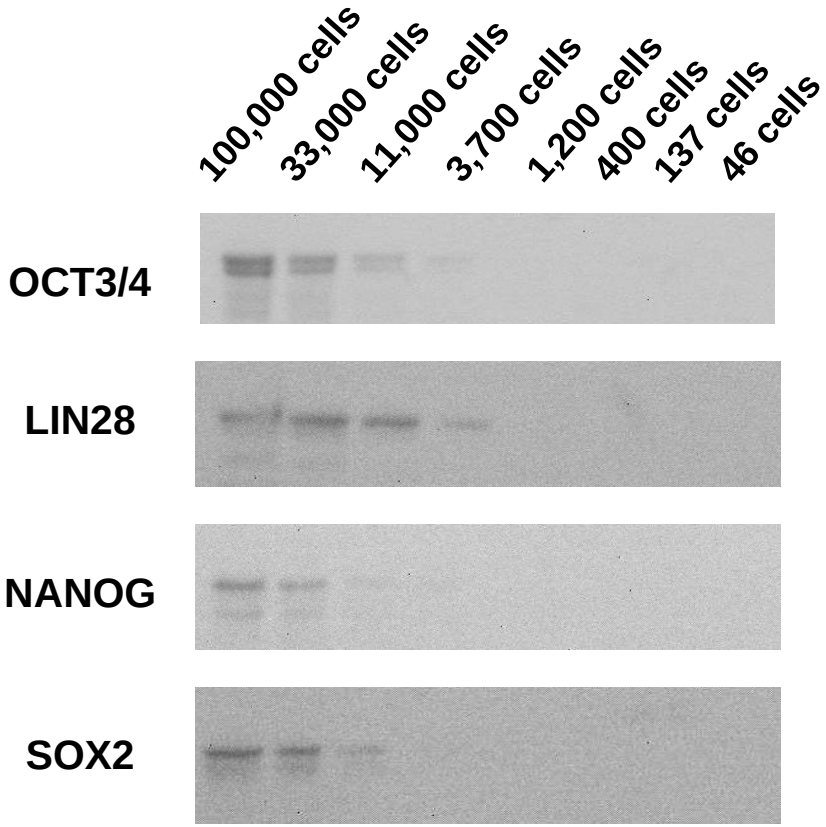


LIN28



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Western vs. TaqMan® Protein Expression Assay



NTERA2 embryonal carcinoma

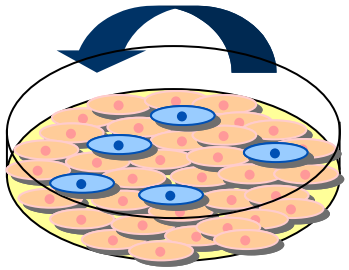
	Western	TaqMan Protein Expression Assay
Typical input	100,000 cells/lane	100-500 cells/well
Limit of Detection	30,000- 10,000 cells/lane	10-35 cells/well
Time	1-2 days	3 ½ hr
Workflow	Tedious, multiple steps	Simple, no wash steps
Output	Gel Image	Numerical
Quantitation Method	Usually +/-	Relative Quantitation

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Protein Markers Assayed in Pluripotent and Mesenchymal Stem Cell Lysates

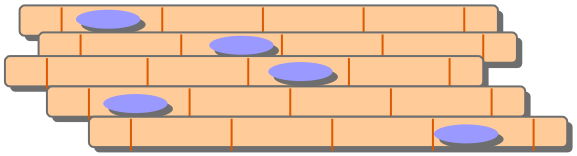
Pluripotent cells:
Self Renewal

SA002



Loss of Pluripotency:
Differentiation

MP002

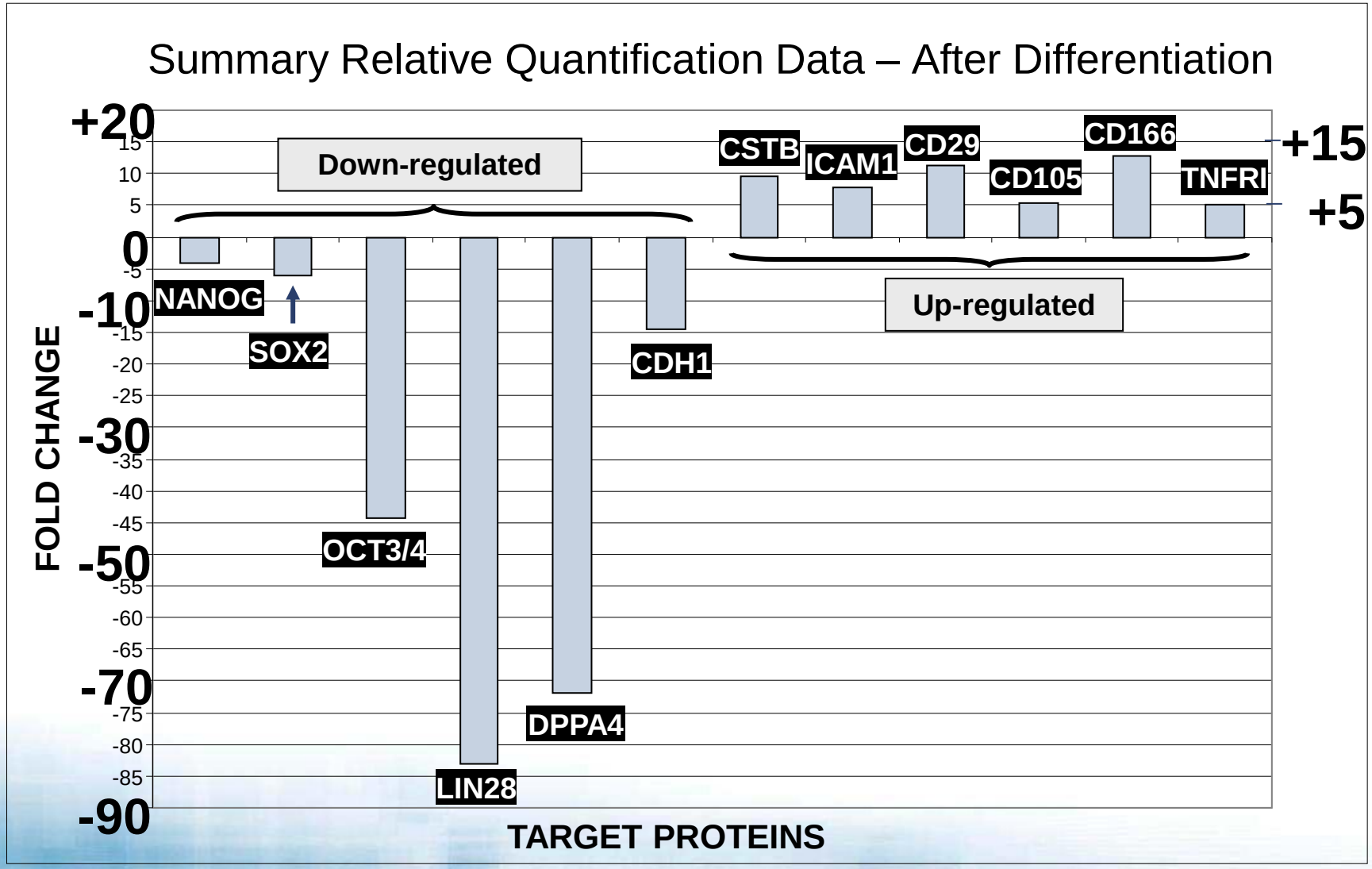


Mesenchymal cells

Key Markers ↓	Key Markers ↑
LIN28	CSTB
NANOG	ICAM1
OCT3/4	CD29
SOX2	CD105
DPPA4	CD166
CDH1	TNFR1

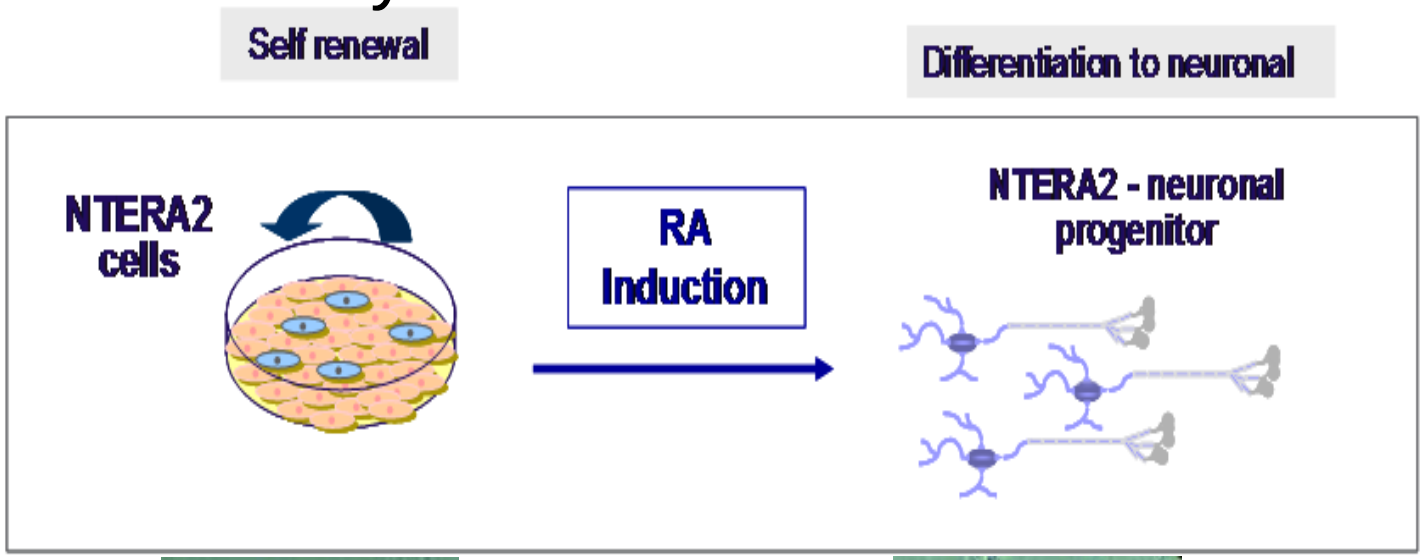
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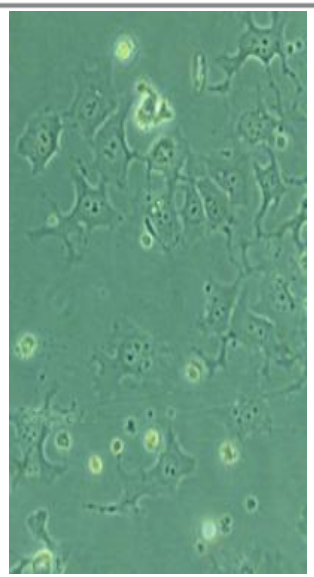
TaqMan® Protein Expression - Differentiated/Pluripotent



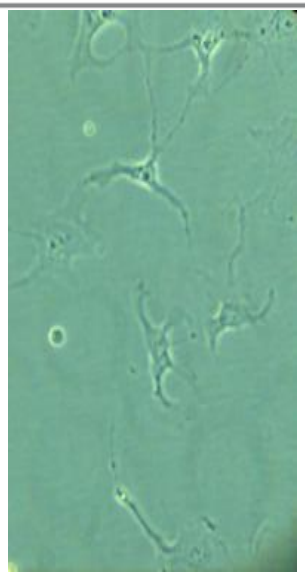
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Analysis of protein and RNA expression: NTERA2 early neuronal differentiation model





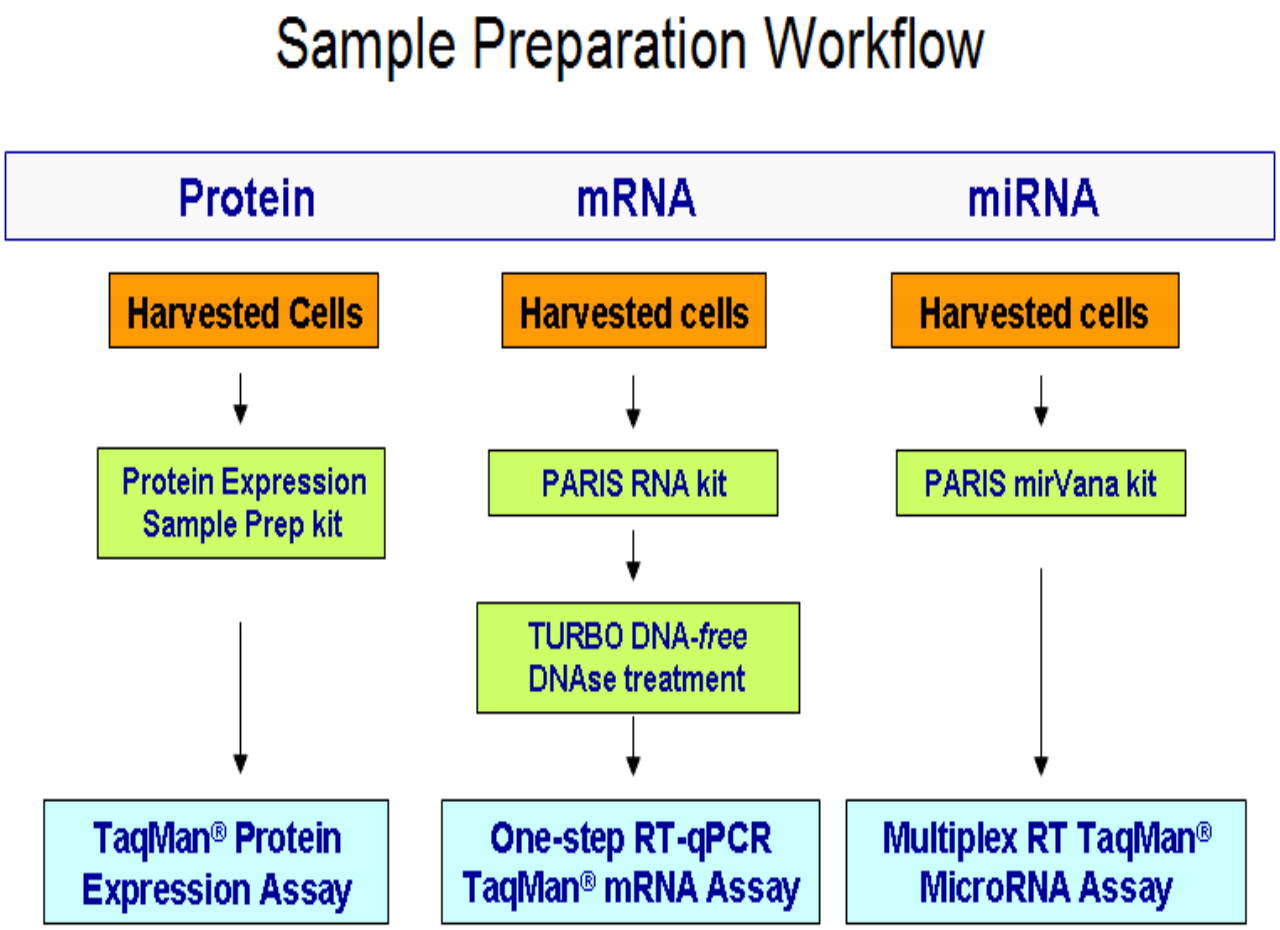
Day 10
(-) RA



Day 10
(+) RA

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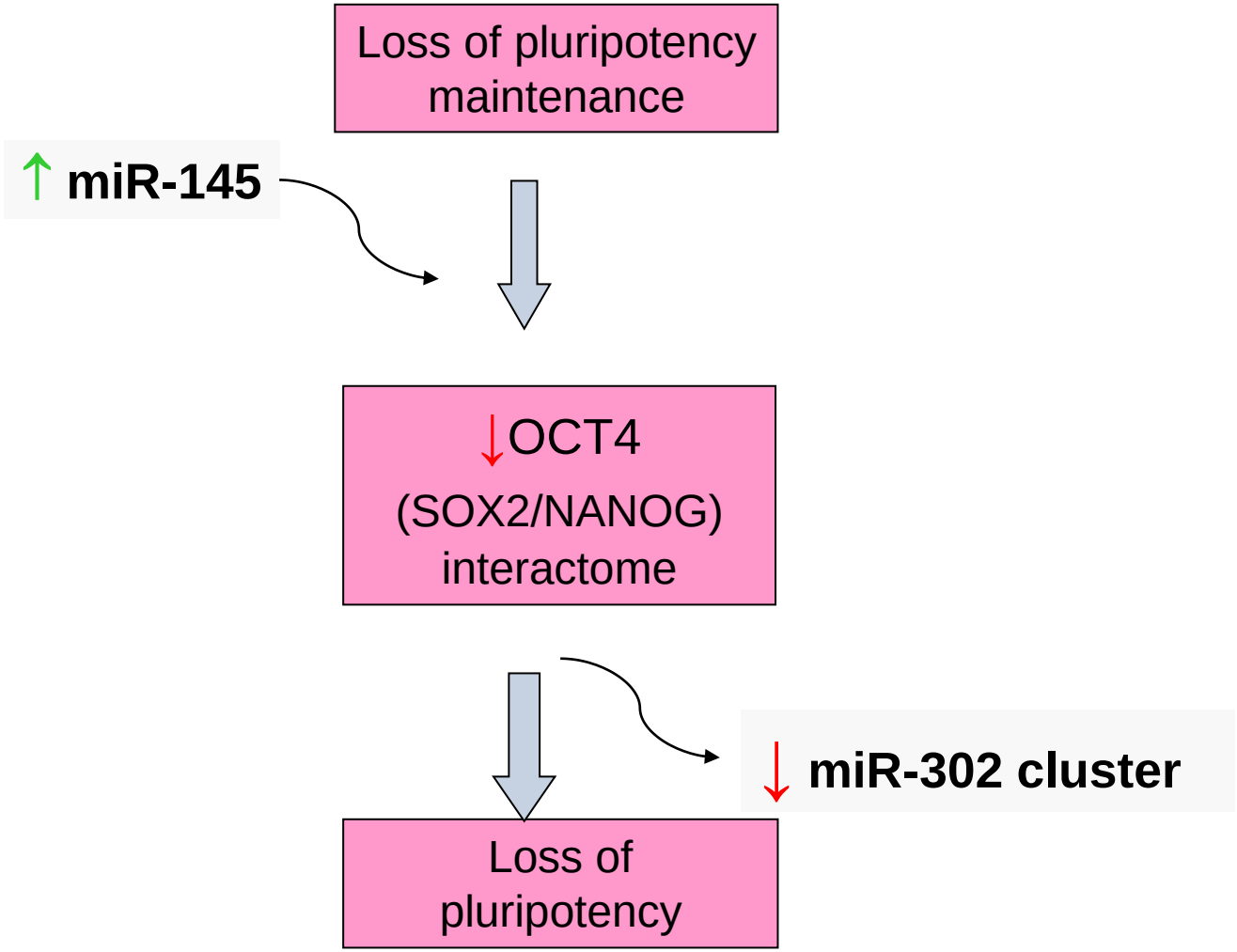
NTERA2 cell lysate workflows



Harvest and assay at day time points 0, 1, 4, 10 and 14

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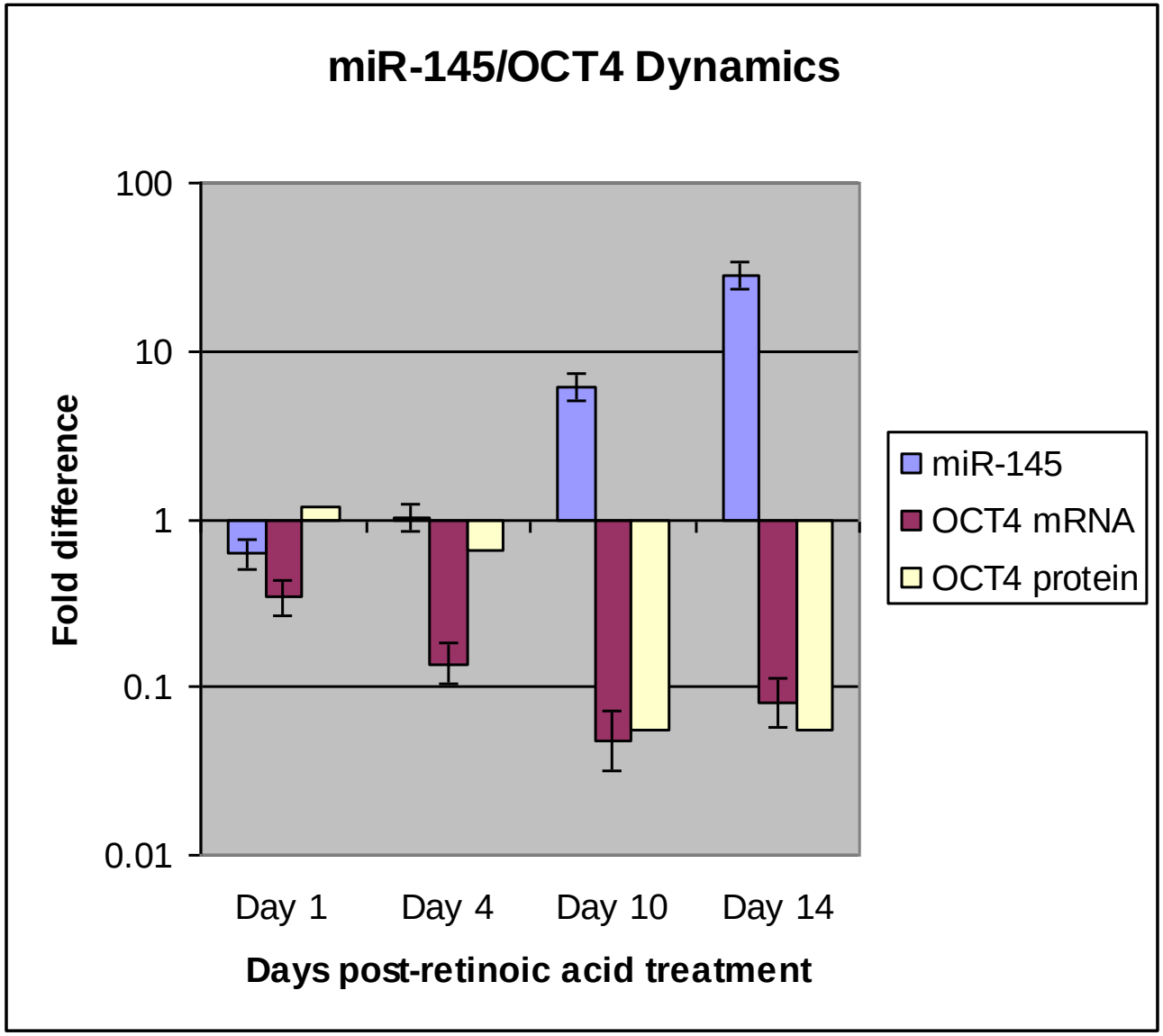
NTERA2 Loss of Pluripotency:
miR-145 and OCT4 expression changes



MicroRNA-145 Regulates OCT4, SOX2, and KLF4 and Represses Pluripotency in Human Embryonic Stem Cells
Xu, N. et. al. Cell 137, 647-658 May 15, 2009

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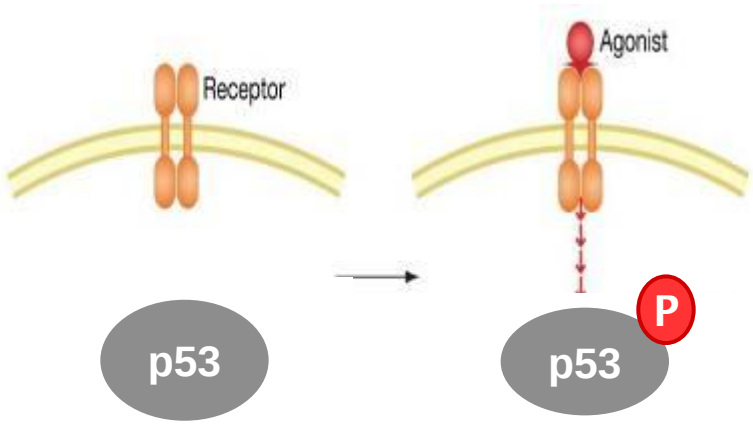
NTERA2 Loss of Pluripotency:
miR-145 and OCT4 expression changes

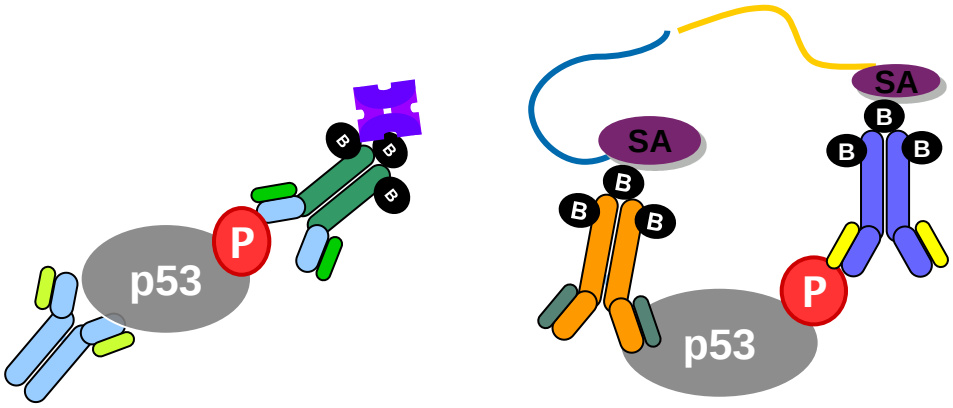


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TaqMan® Protein Assays for Phosphorylation

Endogenous p53 pSer15 in cell lysates





ELISA kit

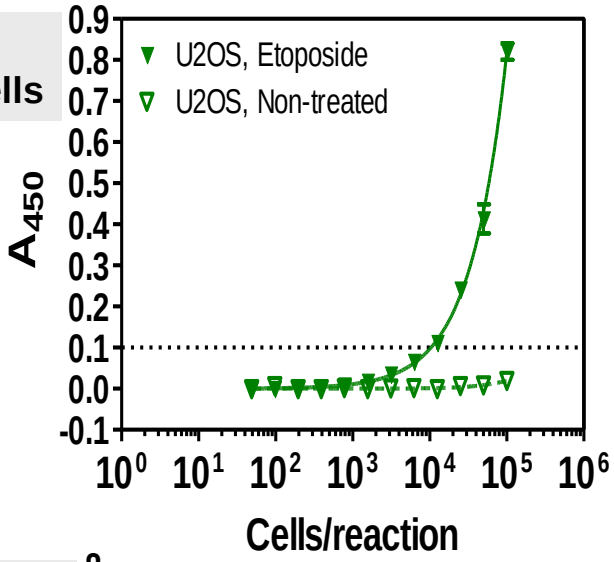
mAb1, mAb2

Protein Assay

pAb1, mAb2

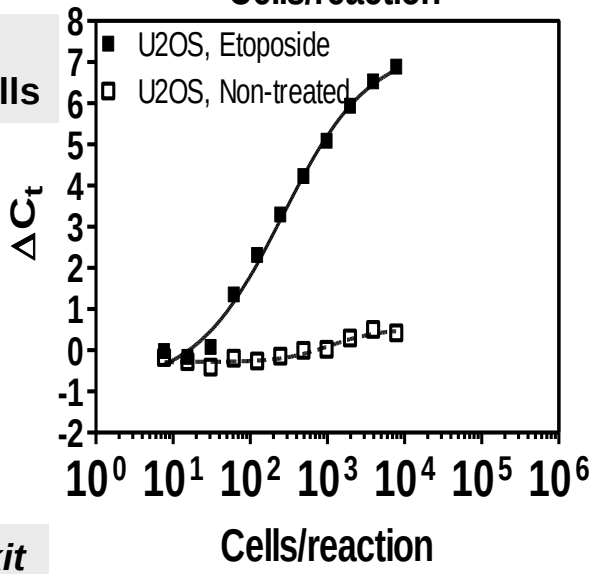
ELISA

LOD 1600 cells



Protein Assay

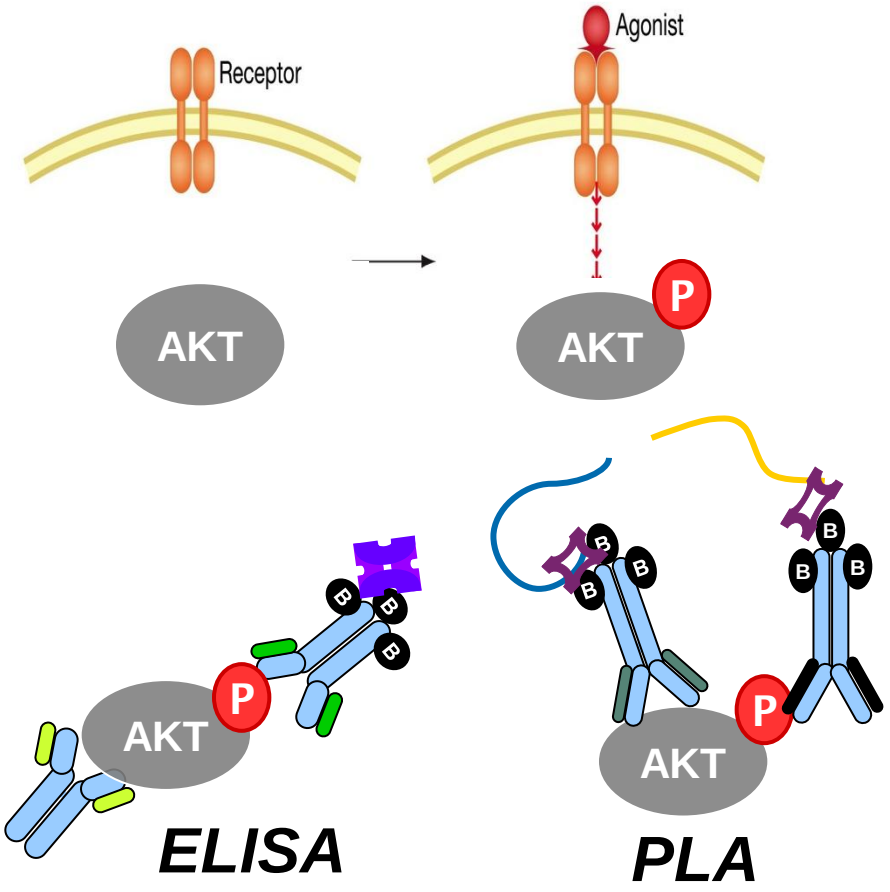
LOD 125 cells

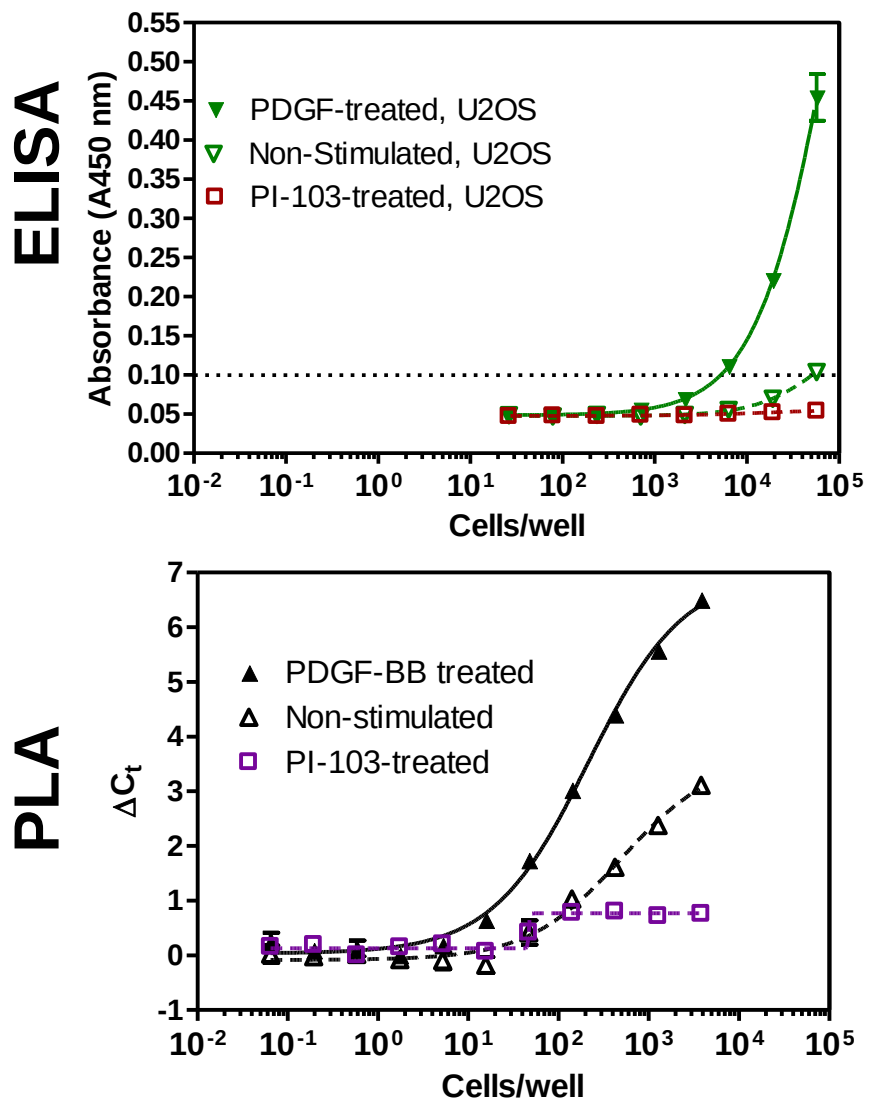


p53 pSer15 PLA - more sensitive & greater dynamic range vs. ELISA kit

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Benchmarking endogenous AKT pSer473 in cells





LOD based on Z' >0.5:

ELISA-stim	6,300 cells/well
PLA-stim	50 cells/well

AKT pSer473 PLA developed was ≥50-fold more sensitive than ELISA

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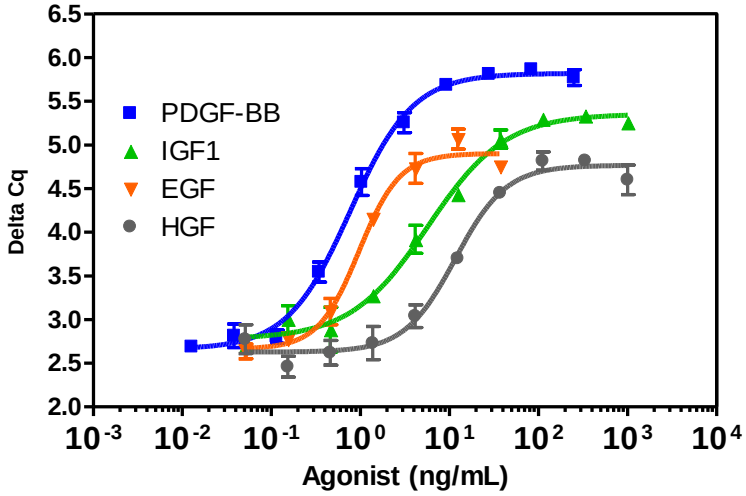
Pharmacology for pS473 AKT: ELISA v. PLA®

- U2OS starved prior to stimulating with dose responses of 4 agonists for 14 min at 37ºC prior to cell lysis.

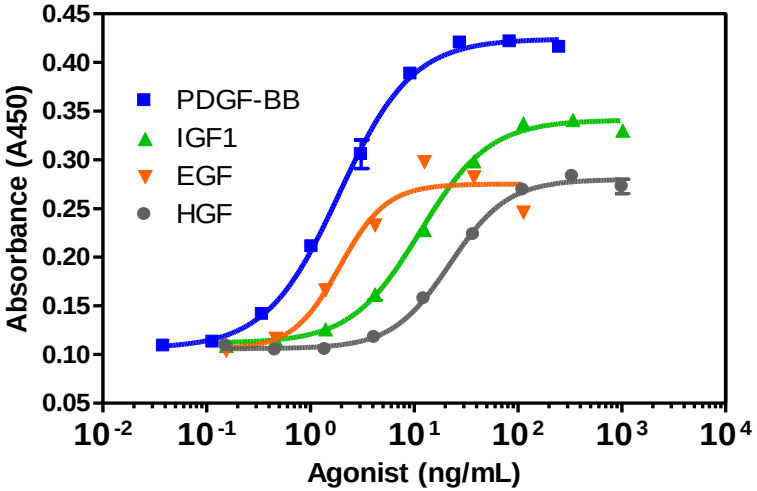
- ***Pharmacology obtained with PLA® comparable to ELISA using 30-fold fewer cells***

		AKT pS473 EC_{50} (nM)	
	Compound	PLA **750 cells/well**	ELISA **22,500 cells/well**
■	PDGF-BB	0.77 nM	1.9 nM
▲	IGF1	5.7 nM	11 nM
▼	EGF	0.96 nM	1.9 nM
●	HGF	12 nM	22 nM

PLA pSer473 AKT: Equivalent of 750 cells/well



ELISA pSer473 AKT: Equivalent of 22,500 cells/well



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Summary:

- PLA® / TPA technology enables a broad range of protein detection applications, including quantification of:
  - Proteins in cells (including single cells) and tissues
  - Protein modifications
  - Protein-protein interactions
  - Serum/plasma biomarkers
- The simple workflow is ideally suited for high-throughput screening applications
  - All assay steps are completed in a single plate and are compatible with automation

Concluding Remarks:

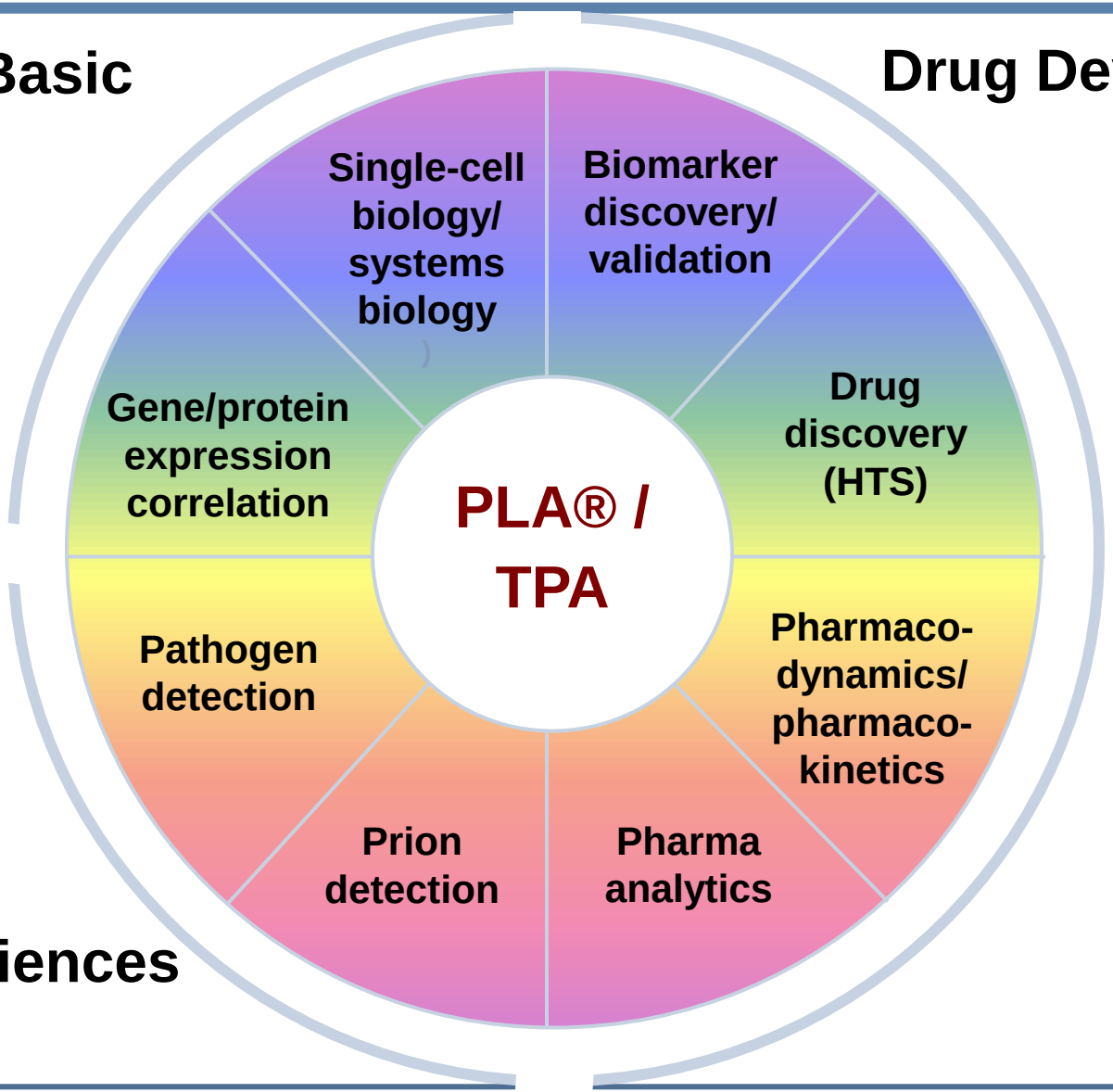
- PLA® /TPA offers a simple and flexible platform – homogeneous and solid-phase formats
- PLA® is scalable
  - # of assays - Singleplex or multiplex formats
  - # of samples - High throughput screening
- Enabling multi-analyte detection from a single sample on a single platform – DNA, RNA and protein
- PLA® has a low sample input requirement (1-2μL)

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Proximity Ligation Assay Application Areas

Genomic/Basic Research

Drug Development



Health and Medical Sciences



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"75% of protein research still focuses on the 10% of proteins that were known before the genome was mapped – even though many more have been genetically linked to disease."

—Edwards AM et al, Nature. 470,163-165 (2011).

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Acknowledgements

Technical Development

Mark Shannon
Shiaw-Min Chen
David Ruff
David Joun
Jane Kuo
Elana Swartzman
Julia Lu
Jack Tian
Kristin Huwiler
Erica Graziosa
Junko Stevens

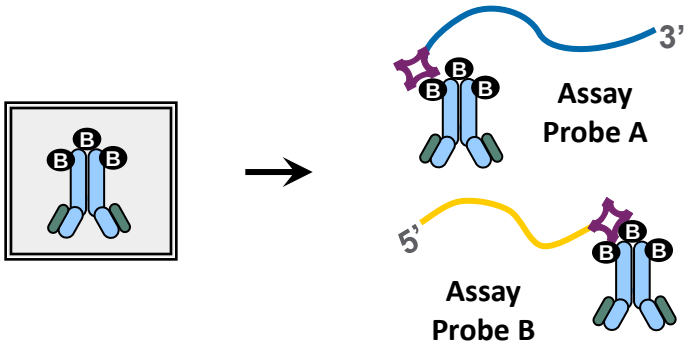
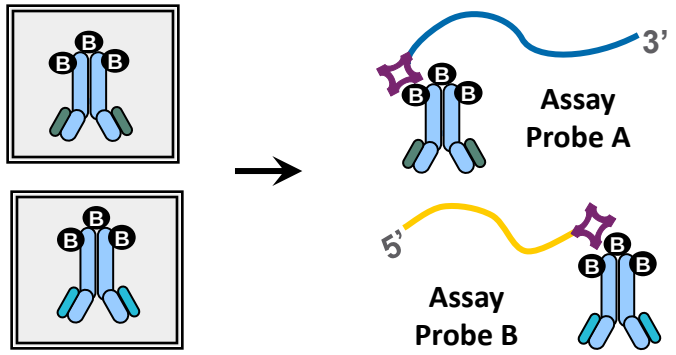
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Appendix Slides

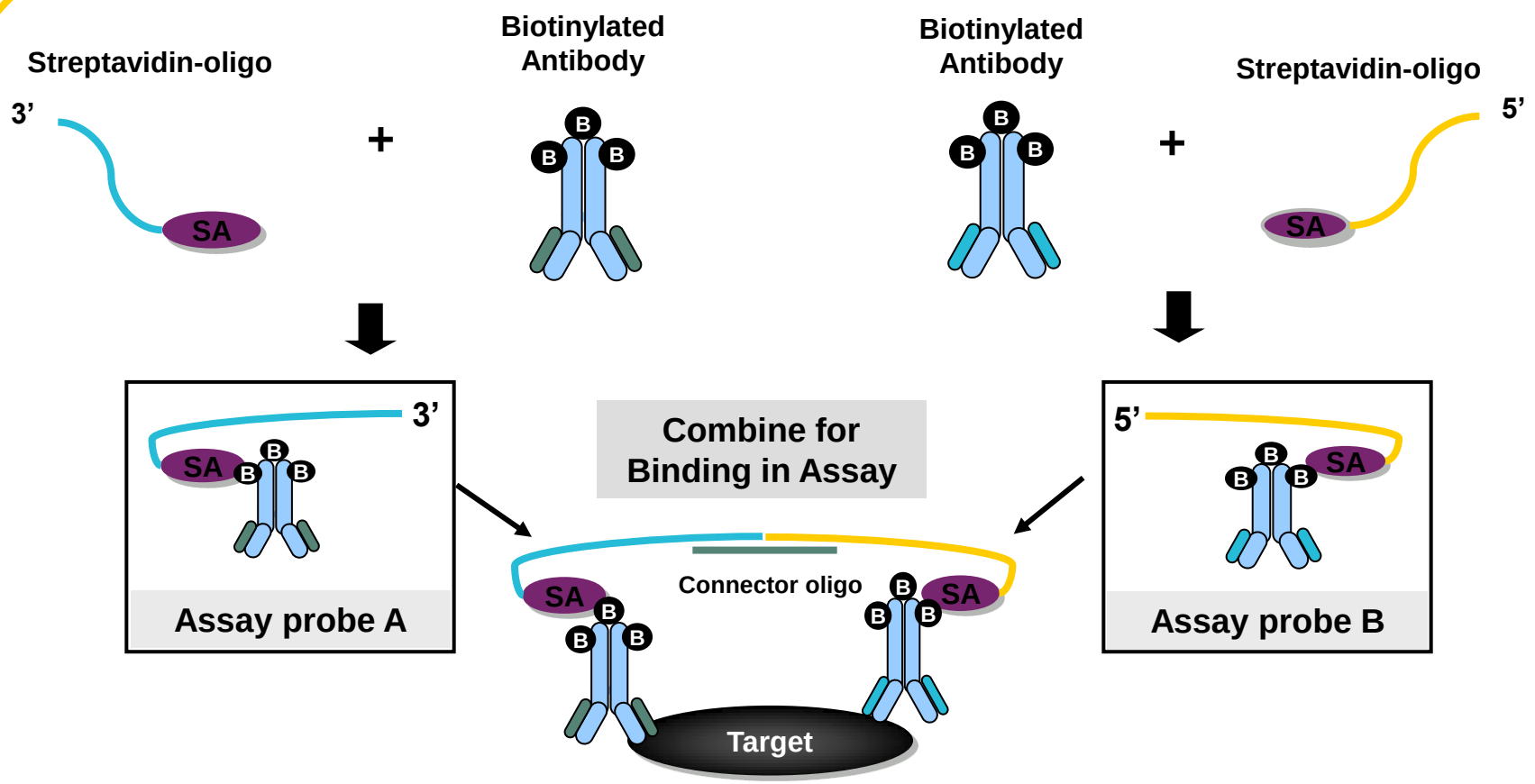
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Design Elements: The “Binders”

Polyclonal Antibody	•Antigen (affinity) purified •Raised against full length or partial antigen (> 100 aa); polyclonals to peptides not extensively tested	
Monoclonal Antibody	•Often used successfully in pairs or in combination with a suitable polyclonal when qualified by ELISA	
Aptamers	•Single aptamers are suitable for detection of homo-dimers, homo-trimers, etc… •Development of aptamer pairs could be challenging •Not ideal for detecting modifications such as phosphorylation	

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Design Elements: Making Proximity Probes



Antibodies typically 2 *different* mAbs, 1 mAb + 1 pAb, or a split pAb

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Design Elements: Assay Buffers and Reagents

- Sample preparation
  - Non-denaturing reagents for cell lysis or tissue homogenization (recommended)

	Applied Biosystems (kits developed for TaqMan Protein® Assays)		Invitrogen							Biochain
Product	Protein Expression Sample Lysis Kit	Protein Quant Sample Lysis Kit	Cell Extraction Buffer	NP40 Cell Lysis Buffer	Denaturing Cell Extraction Buffer	Tissue Extraction Reagent I	NativePAGE™ Sample	Paris™	mirVana™ Paris™	Total Protein Extraction Kit: TM Buffer
Part number	4405443	4448536	FNN0011	FNN0021	FNN0091	FNN0071	BN2008	AM1921	AM1556	K3011010-1
Initial dilution †	1:2	1:10	1:10	1:10	1:10	1:10	1:10	1:10	1:10	1:10

† This is the initial dilution required before using the lysate in the TaqMan® Protein Assays experiment.

- Binding buffers – containing blocking agents (for non-specific protein and nucleic acid binding and matrix effects)
  - Probe dilution buffer
  - Sample dilution buffer
- Ligation/PCR reagents – fluorescent probe-based chemistry (recommended)

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Data Analysis Considerations

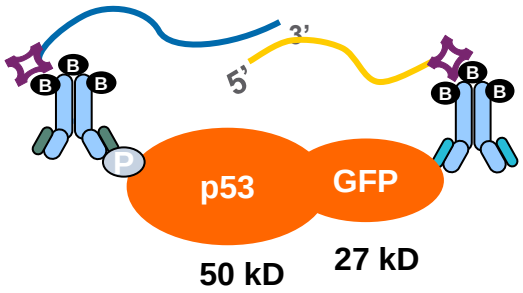
- Type of quantification
  - Relative quantification (RQ)
    - Use of sample dilution curves
    - “Single point” RQ using reference assay for normalization
  - “Absolute” quantification
    - Use of standard curves for quantification

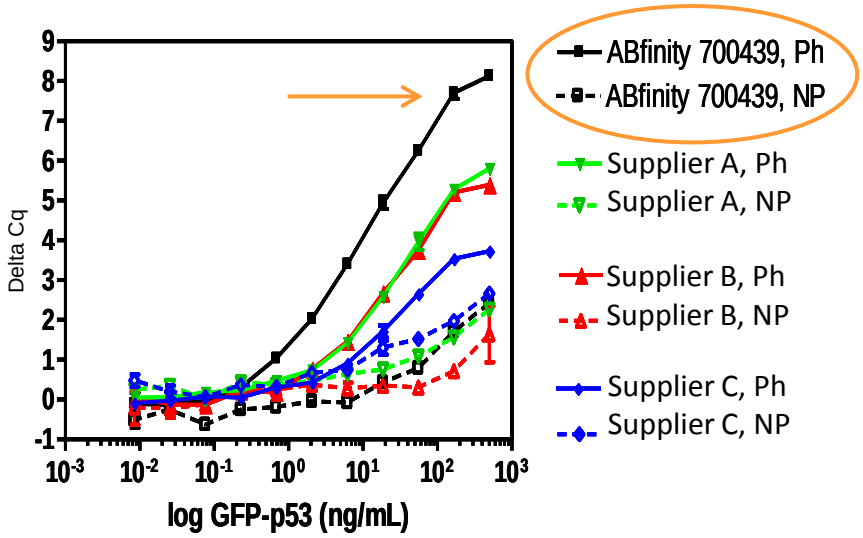
- Normalizing for sample input (unit of measure)
  - Cell count – hemocytometer, Countess® cell counter
  - gDNA as a surrogate for cell count (singleplex or duplex reactions)
  - Total protein – determined by microBCA™ protein assay kit (ThermoFisher, PN 23235) or similar method
  - Unit volume

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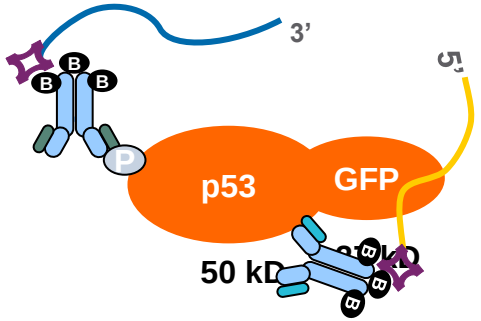
Using Different Antibodies and Combinations

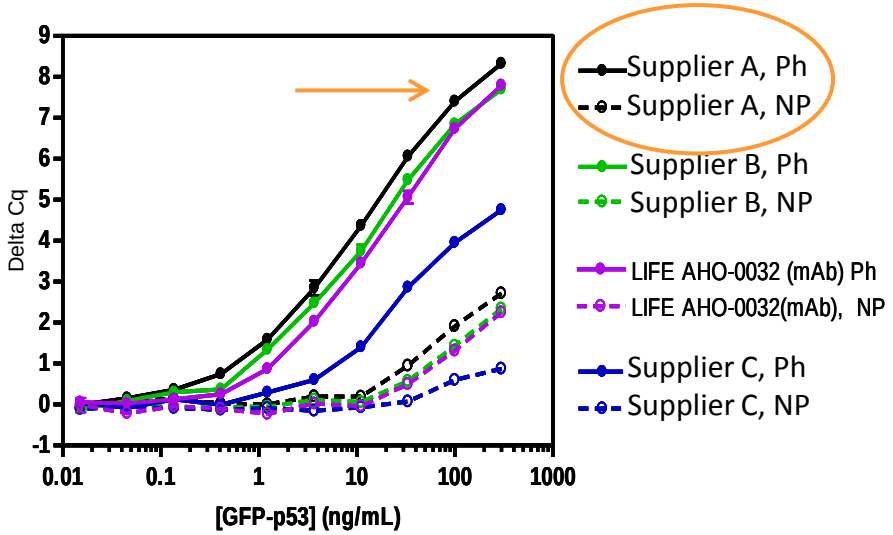
pSer15 Antibody Screen:
pSer15-p53 Abs v. fixed GFP pAb





Pan p53 Antibody Screen:
Abfinity® p53 pSer15 v. pan p53s





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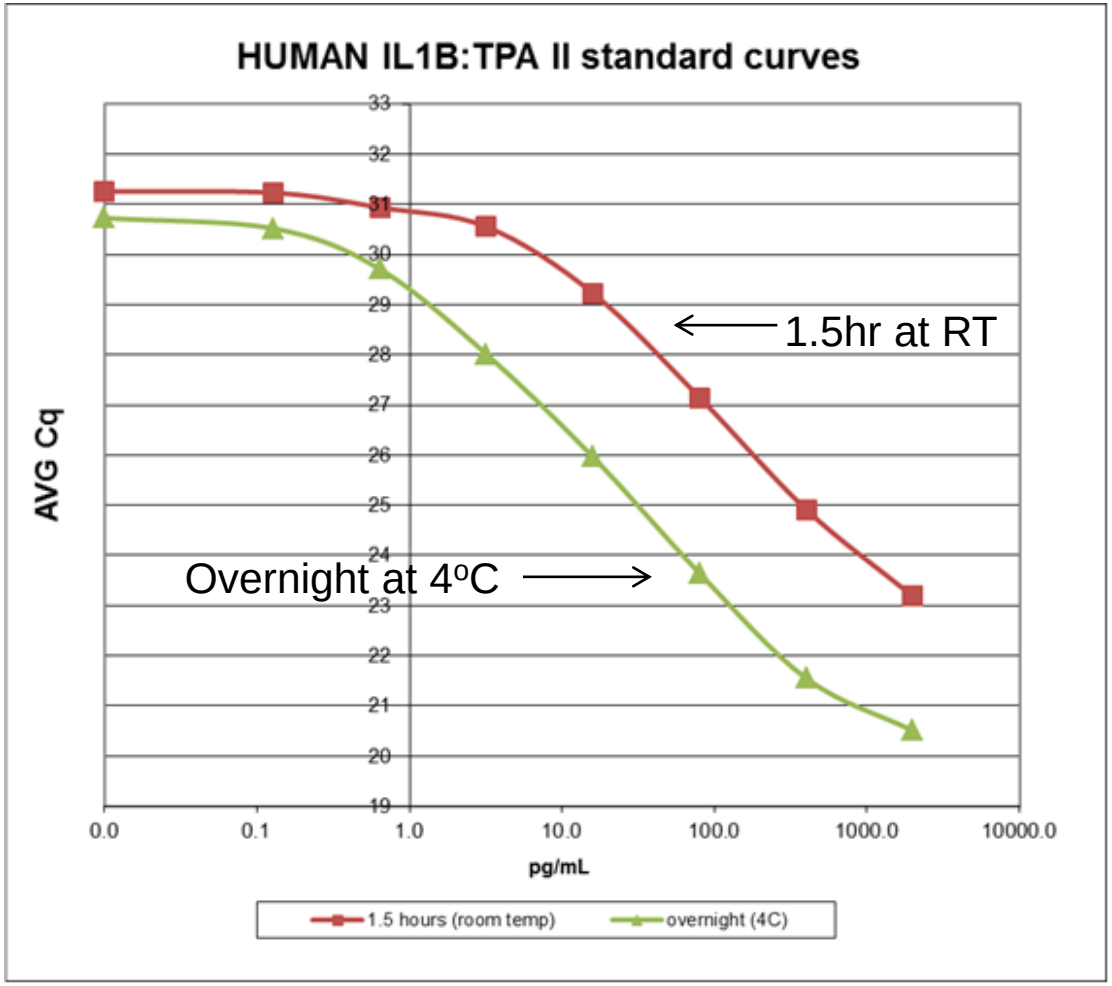
Assay Optimization Parameters

- Incubation time and temperature
  - 1-2 hour binding at room temperature is often sufficient
  - Overnight at 4°C can improve performance

  appreciably for some assays
- Probe concentration
  - 50-200pM probes is recommended
- Trying different antibodies and combinations is highly recommended
- Probe orientation may influence assay performance
- Sample buffer composition may be critical for optimal assay performance
  - Sample or buffer components can inhibit assay or cause false signal
  - Sample or buffer components can effect assay linearity

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Effect of Binding Time and Temperature

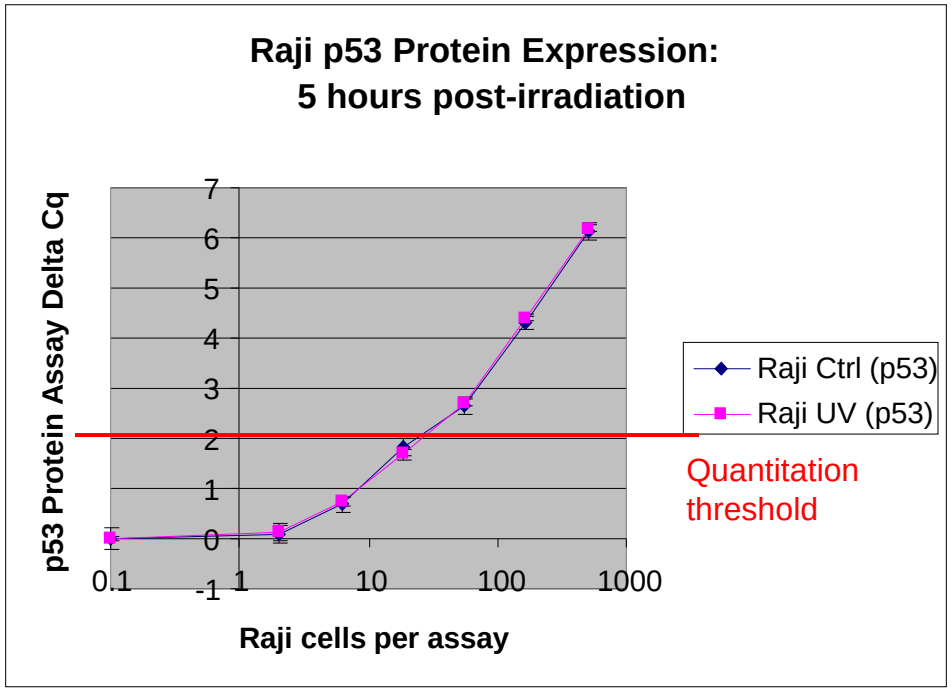


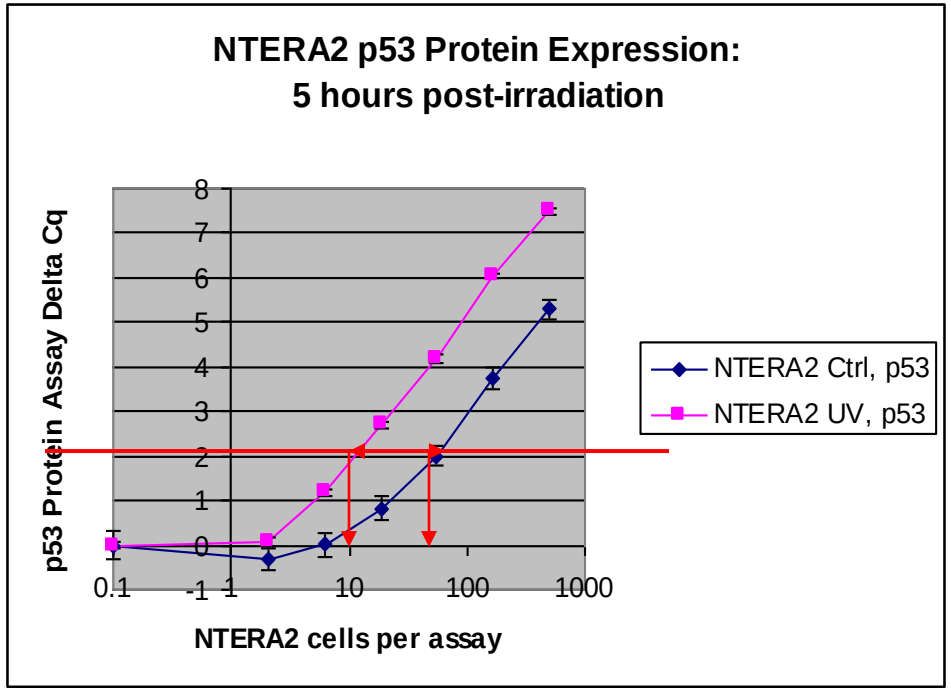
~10-fold improvement in assay performance with binding at 4ºC overnight

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Relative Quantification: Normalization to Cell Input

ProteinAssist™ Software





p53 no change

p53 RQ cell-intercept: 56/10 cells
5.6 fold induction

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