Applications of HTRF and Tag-lite Assays for HTP Antibody Screening

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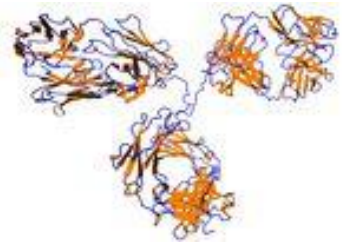
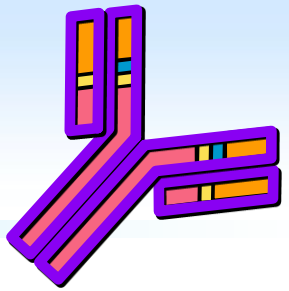


HTRF Symposium

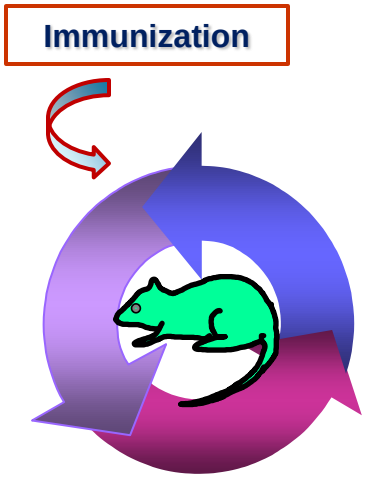
April 25, 2013

Bristol-Myers Squibb

Introduction



- Generate human therapeutic antibodies to large number of targets (soluble or membrane bound) for various therapeutic indications
- Also produce reagent antibodies for research/development/clinical applications
  - Hybridoma technology – Fusion of splenocytes with myeloma partner
- Need to develop HTP assays amenable to screening hybridoma supernatants from fusion plates
  - Create potential for screening on multiple antigens or cell types
    - Tagged antigen versus irrelevant Ag with same tag
    - human/cyno/mouse Ag target
    - Conjugated Ag versus un-conjugated Ag (with PEG for example)
    - Homologues from same family
    - Transfectants and cancer cells



Hybridoma Workflow

Human Ig Tg mice *spleens*

Balb/c *spleens*

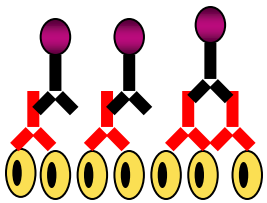
E-Fusion
Splenocytes + fusion partner

HuG/K Screening
HTRF
Automation-96

Antigen Screening

ELISA (soluble Ag)
Hybridoma-96
HTRF (soluble Ag)
AD-384

FACS (cells)
Hybridoma-96
FMAT/Tag-lite (cells)
AD-384



Ag specific Hit Picking
Automation-24

Rescreen of positive lines

Subcloning – Expansion
Purification

HTP Antigen Screening Assays

Requirements:

- Detect antibody binding to antigen (Soluble or Cell based)
- Robust & flexible assays
- No wash steps
- Work in conditioned media
- Sensitivity: Adequate for Ab concentration range from 10 ng-10 μg /ml
- +/- response (no need for EC50 determination)
- Up to 10,000 samples in a run
- Time sensitive (24 hour turn around)
- Rapid assay development against a wide range of targets

Presentation Outline

- **HTRF antigen screening assays (3 examples)**
  - HTRF with tagged antigens (e.g. His, Fc - Ag1 & 2)
  - HTRF with non-tagged antigen (Ag3) and labeled secondary reagents (non-blocking polyclonal)
- **HTRF with multiple labeled antigens in one well (duplexing, 2 examples)**
  - HTRF with Drug Ab IgG4 versus irrelevant IgG4
  - HTRF with PEG versus non PEG Ag
- **HTRF Blocking assay (1 example)**
- **HTRF Tag-Lite (2 examples)**
  - Tag-lite with GPCR1
  - Tag-lite with GPCR2

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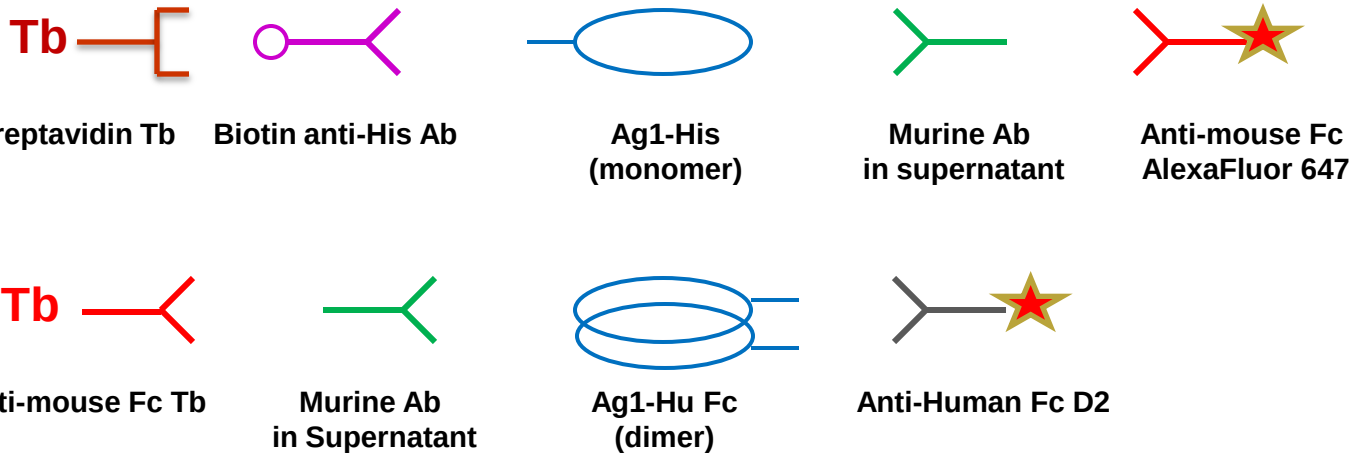
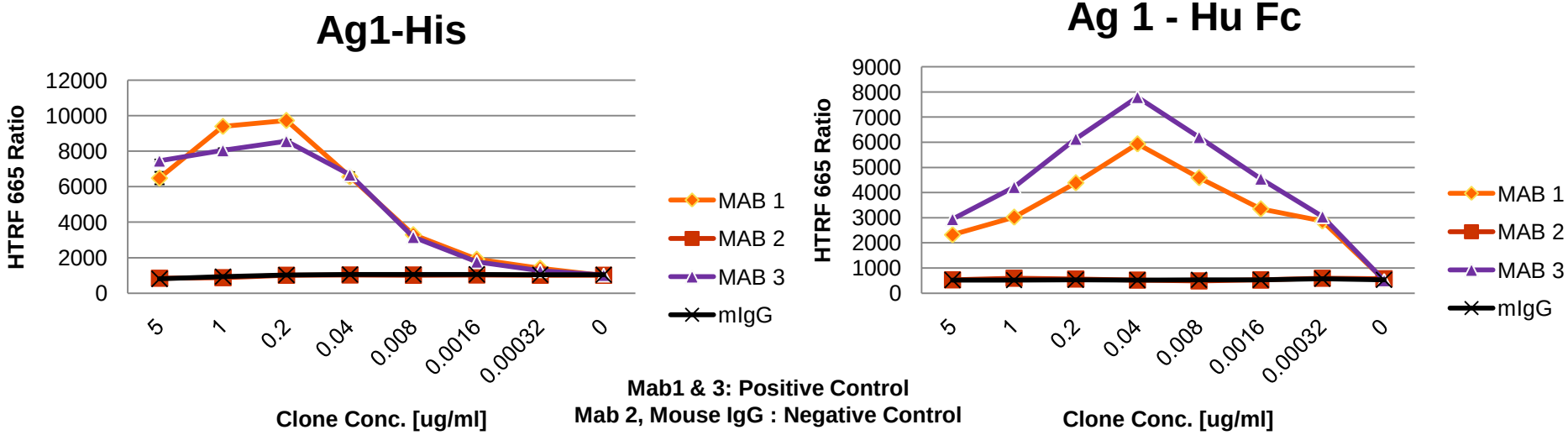
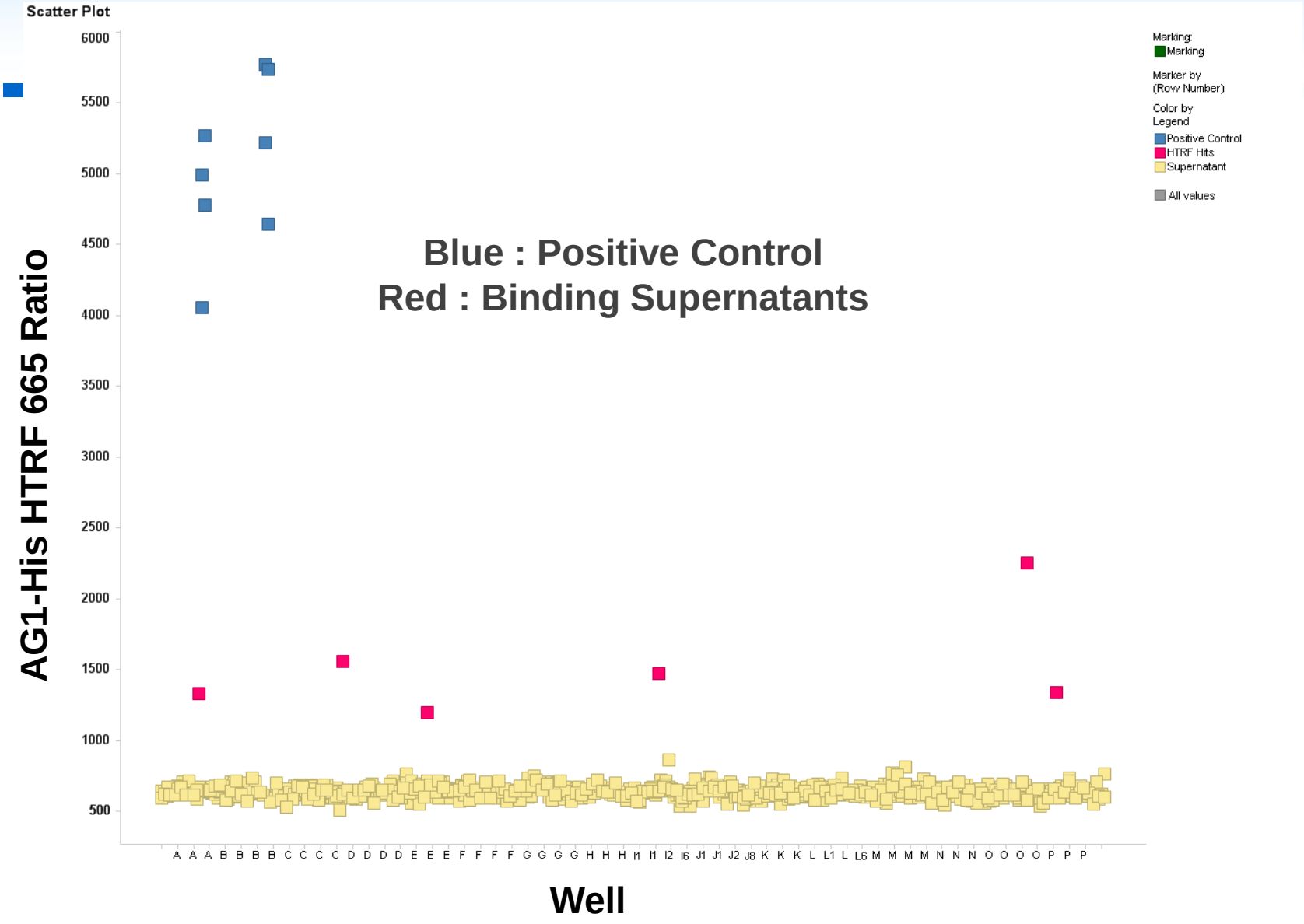
HTRF with Tagged Antigens (His, Fc): Ag 1





HTRF Ag1-His Fusion Screening at 96 well stage



Identification of Six Ab Supernatants with Binding Activity

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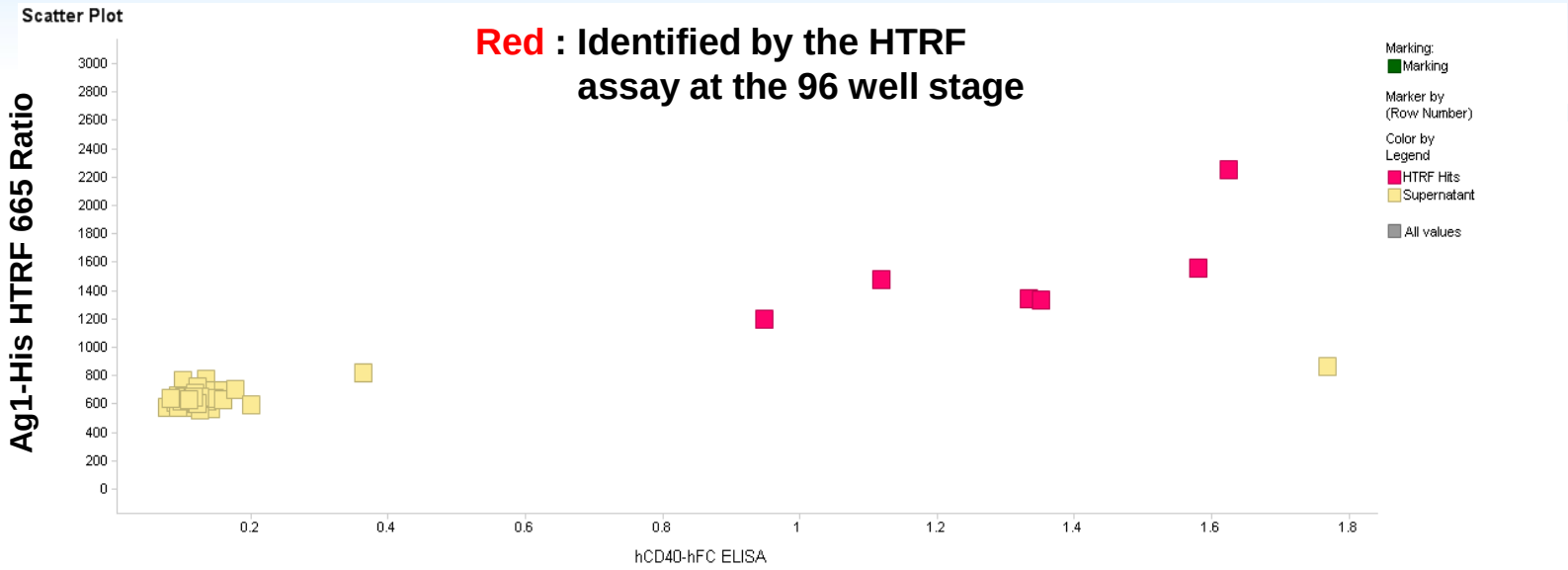
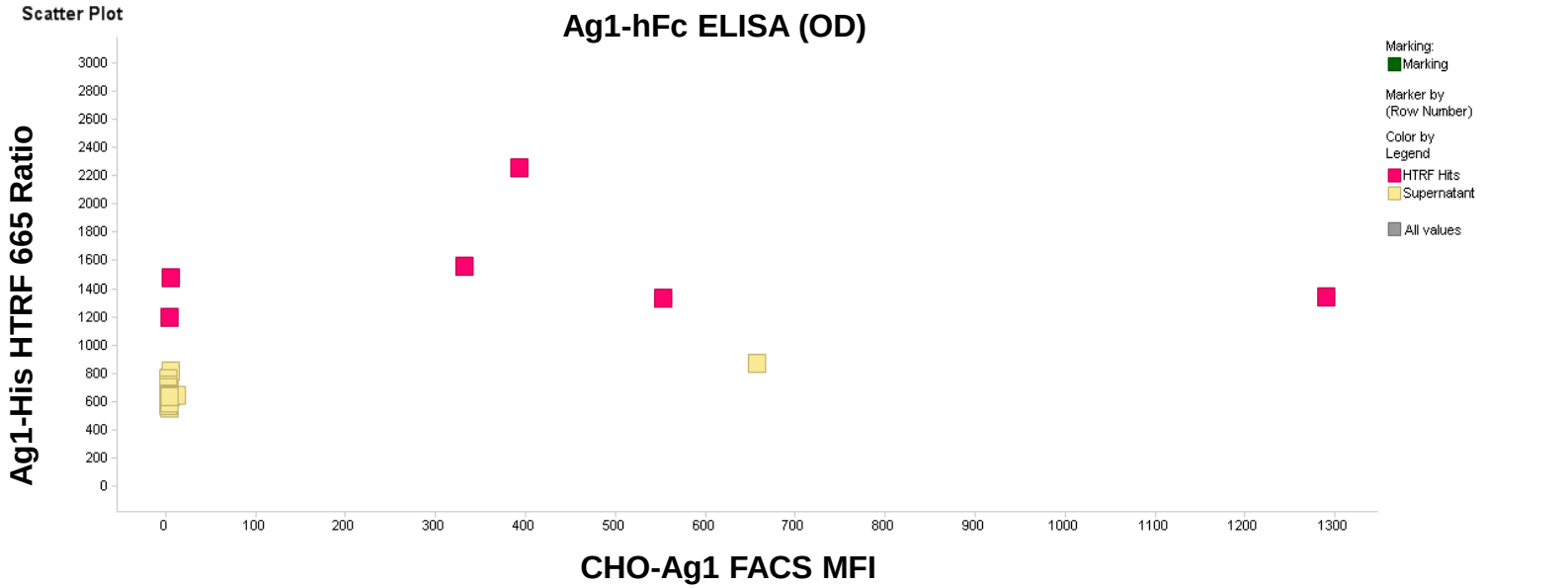
Supernatants tested in Ag1 ELISA and FACS assays at 24 well stage





Same hits in HTRF and ELISA assays
Not all HTRF/ELISA hits bind by FACS

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HTRF with Tagged Antigens (His): Ag 2

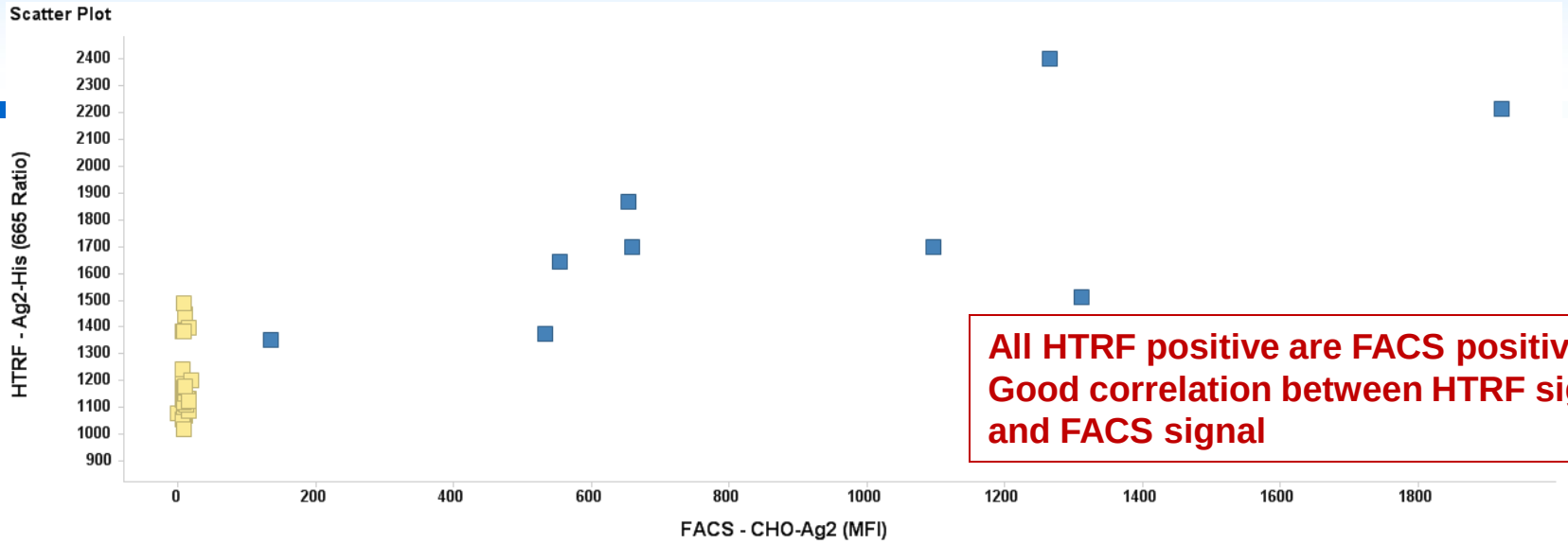
Antigen has a large number of leucine rich repeats on its extracellular surface

Primary screening: HTRF and ELISA in parallel

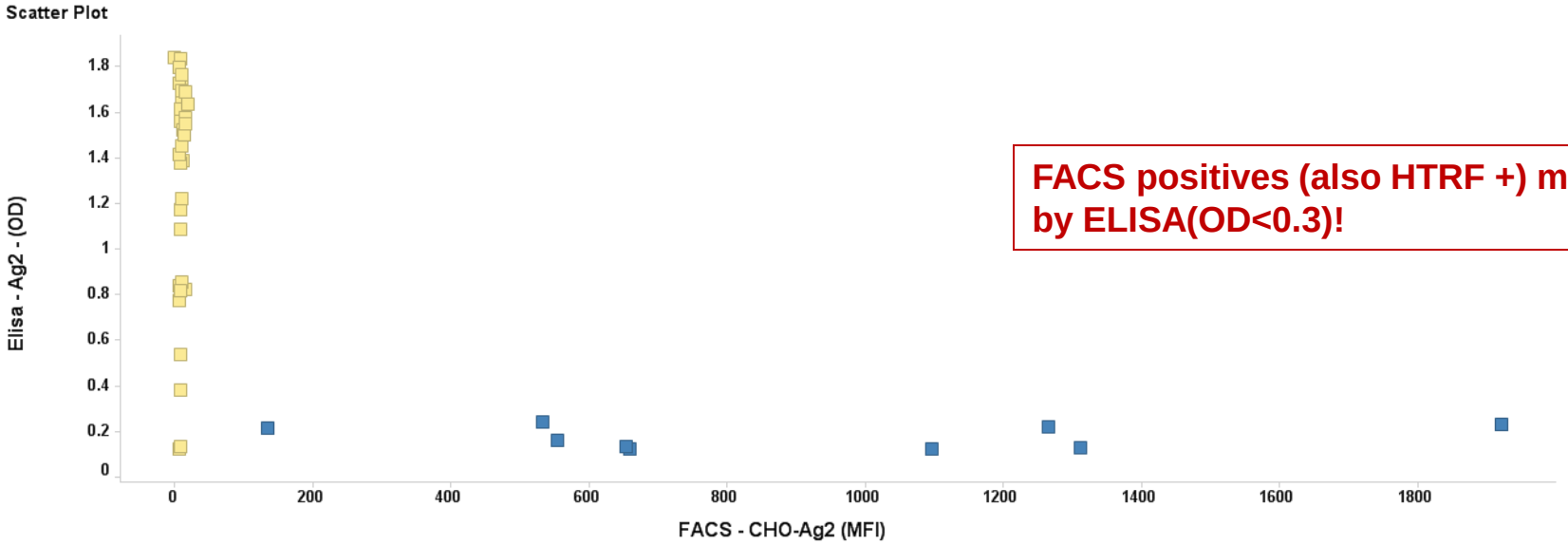
- HTRF binding in solution
- ELISA with Ag coated on plate

HTRF Format

Ag 2 - HTRF & ELISA vs. FACS



All HTRF positive are FACS positives
Good correlation between HTRF signal and FACS signal



FACS positives (also HTRF +) missed by ELISA(OD<0.3)!

Blue are FACS positive - subcloned

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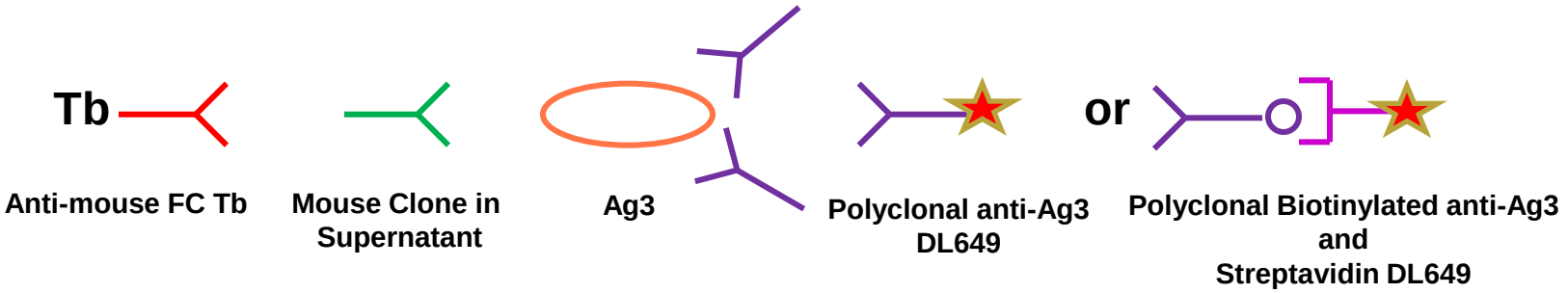
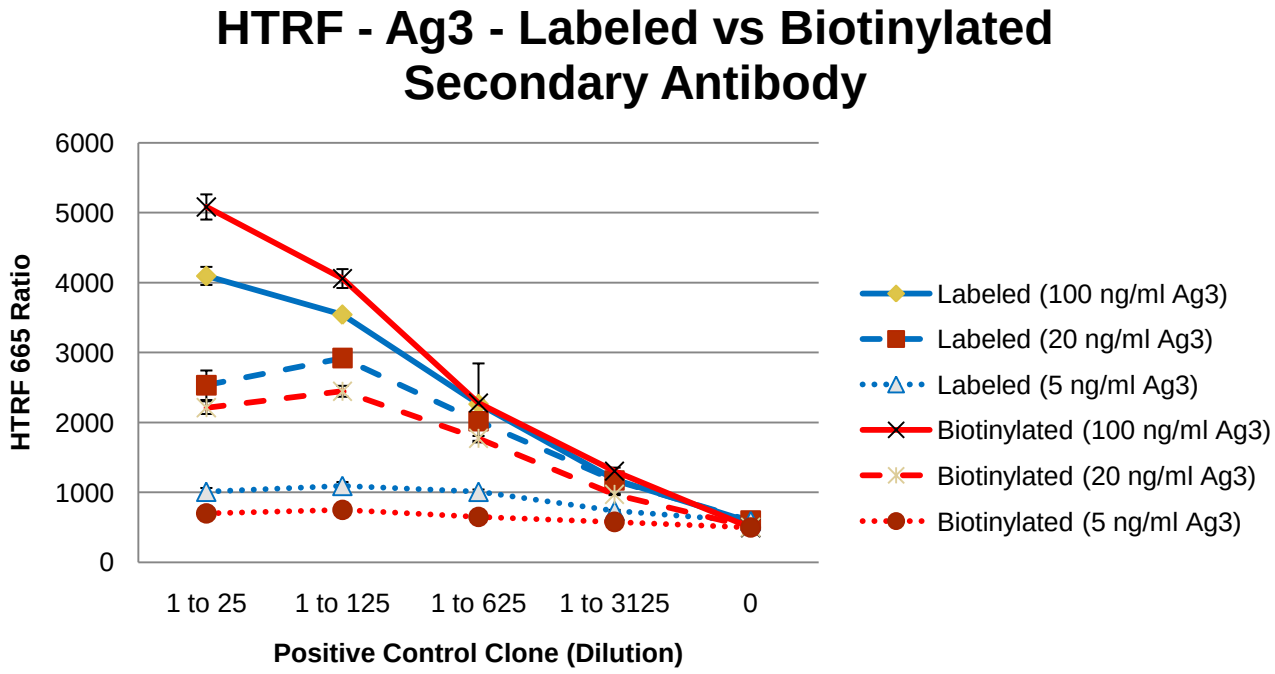
HTRF with Labeled Secondary Reagent Detection: Ag3





Direct and indirect labeling of polyclonal generated same results

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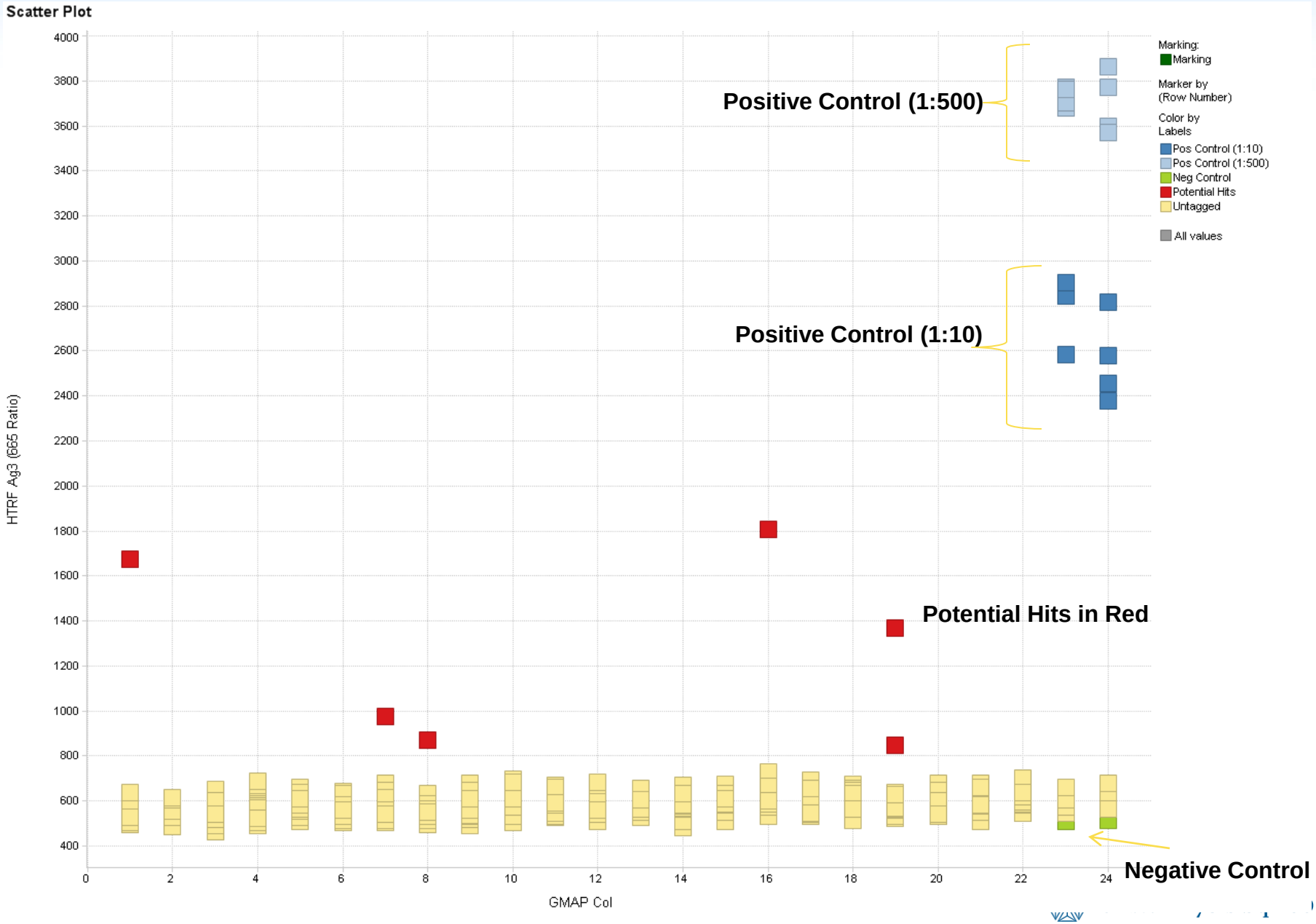
Ag3 – HTRF 650 Ratio



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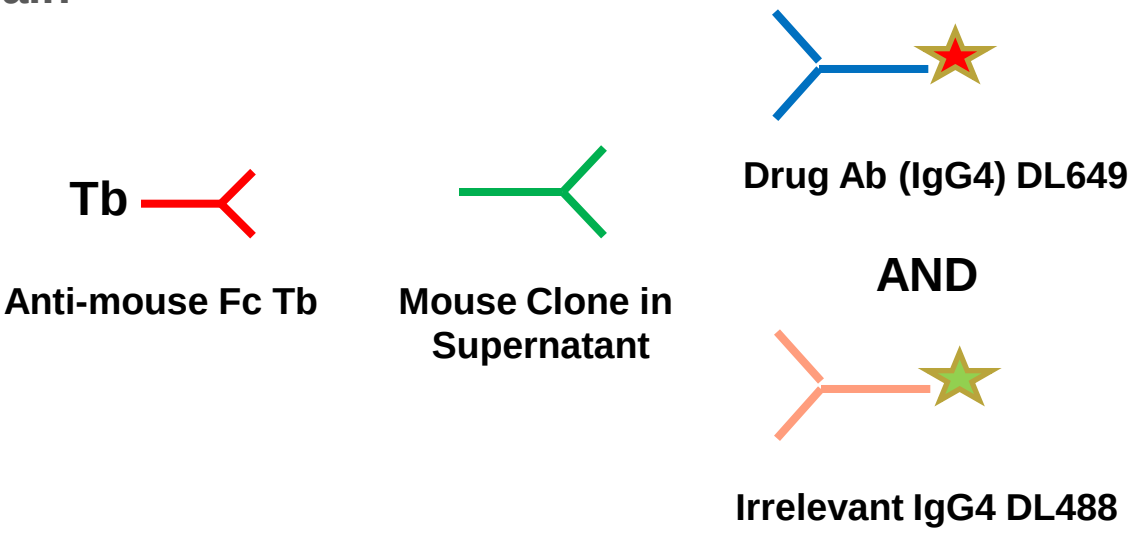
- **HTRF Tag-Lite (2 examples)**
  - Tag-lite with GPCR1
  - Tag-lite with GPCR2

HTRF with multiple Labeled Antigens in one well

Duplexing: Drug Ab IgG4

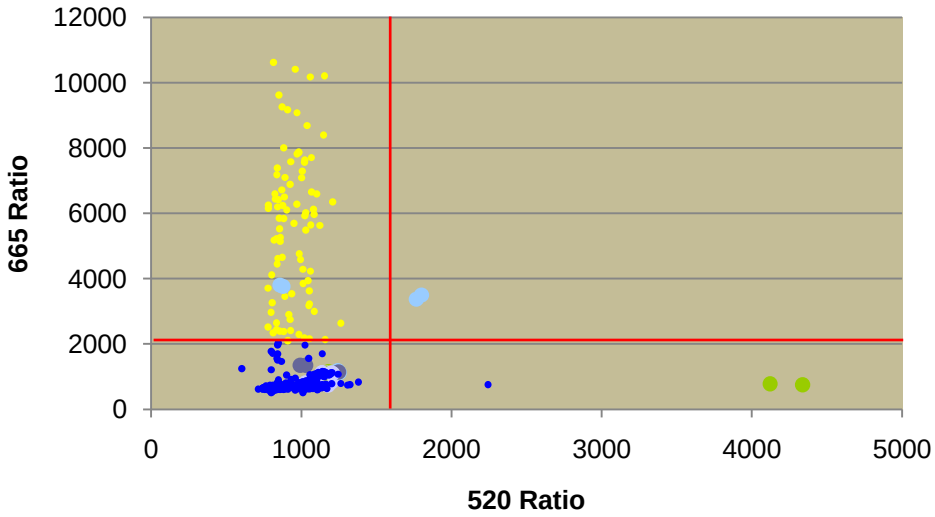
Search for **anti-Id Abs**
The antigen is an Ab!
Do not want Abs binding to Fc domain

"2 assays in 1"

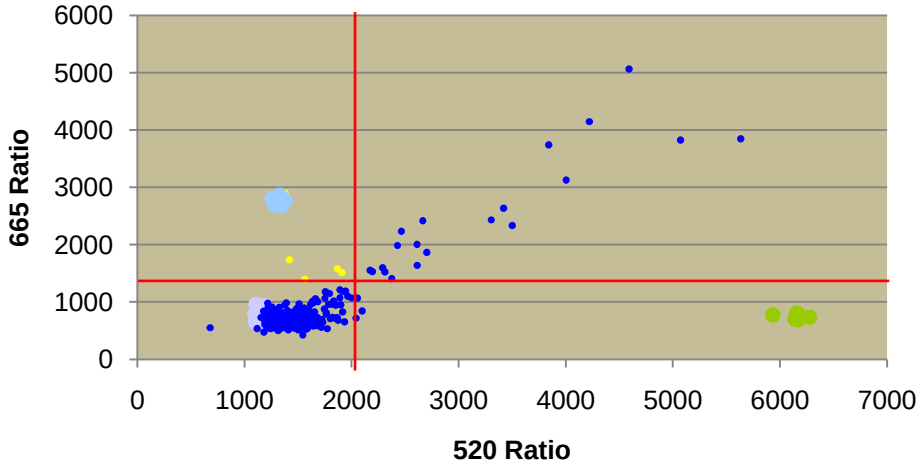


Yellow : Selected hits
Light Blue : Positive Control
Dark Blue or Pink : Not selected

Drug Ab IgG4 DL 649



Immunogen :
Drug Ab V domain only



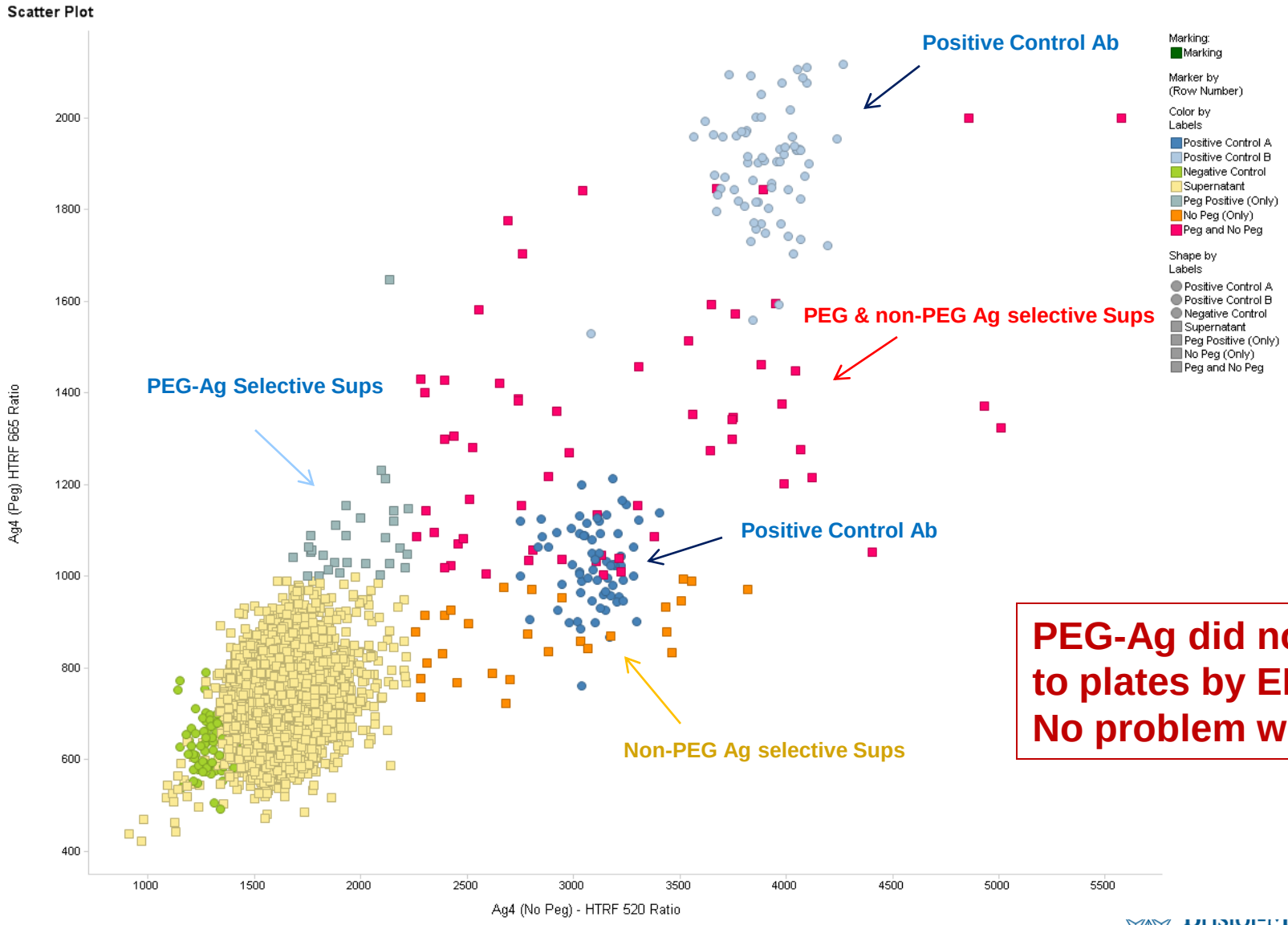
Immunogen :
Drug Ab IgG4

Irrelevant IgG4 DL 488

More anti-Ids obtained when immunizing with V part of the Ab

HTRF with PEG-conjugated Antigen versus non-PEG Ag

Duplexing: PEG-Ag versus non-PEG Ag



PEG-Ag did not coat to plates by ELISA – No problem with HTRF

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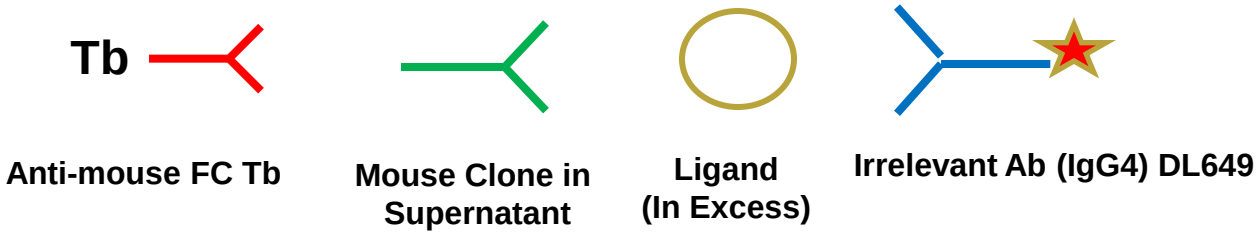
  - Tag-lite with GPCR1
  - Tag-lite with GPCR2

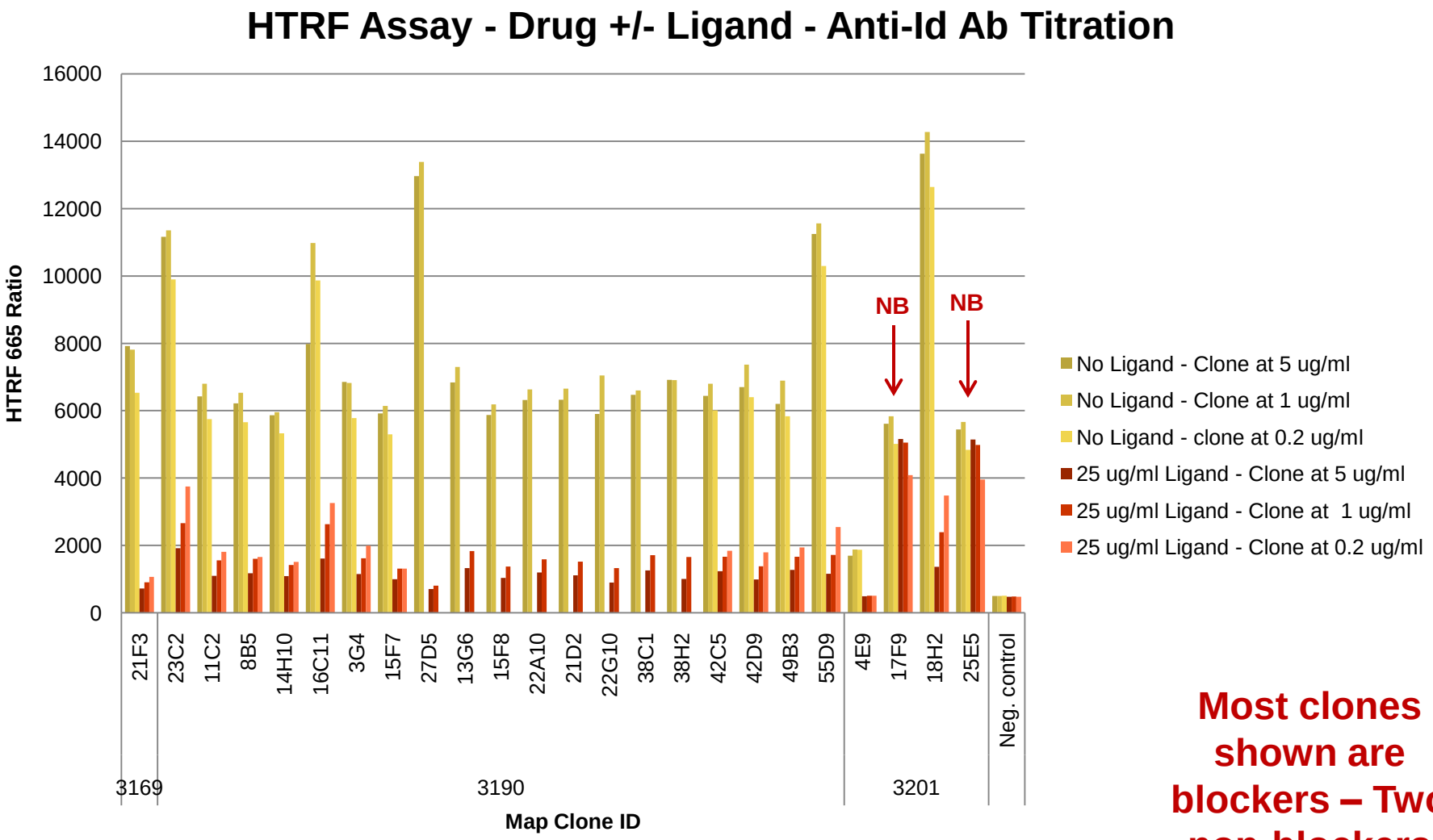
HTRF Blocking assay

Goal: identify anti-Ids which bind to Ab when ligand is bound (non-blockers) or anti-Ids which interfere with ligand binding (blockers)

Or



HTRF Blocking Assay +/- Ligand



Most clones shown are blockers – Two non-blockers (anti-framework)

Preincubation ligand/drug Ab for 45 min

HTRF: Conclusions

Advantages

- Homogenous assay - No wash
- Sensitive (5ng/ml or less)
- Rapid (1 hr incubation time)
- Easily developed and automated
  - Reagents stable
  - Low volume 384 well plates
  - Small reaction volumes
- Binding in solution
  - Binding antigen to plates for ELISA may block a subset of epitopes

Disadvantages

- Homogenous assay – Hook effect
  - Can make distinguishing supernatants based on single point signals difficult
- Requires antigen to be tagged or labeled
  - May remove access to certain epitopes
- Matrix (conditioned media) can interfere with HTRF ratio
  - Ideally positive control in media

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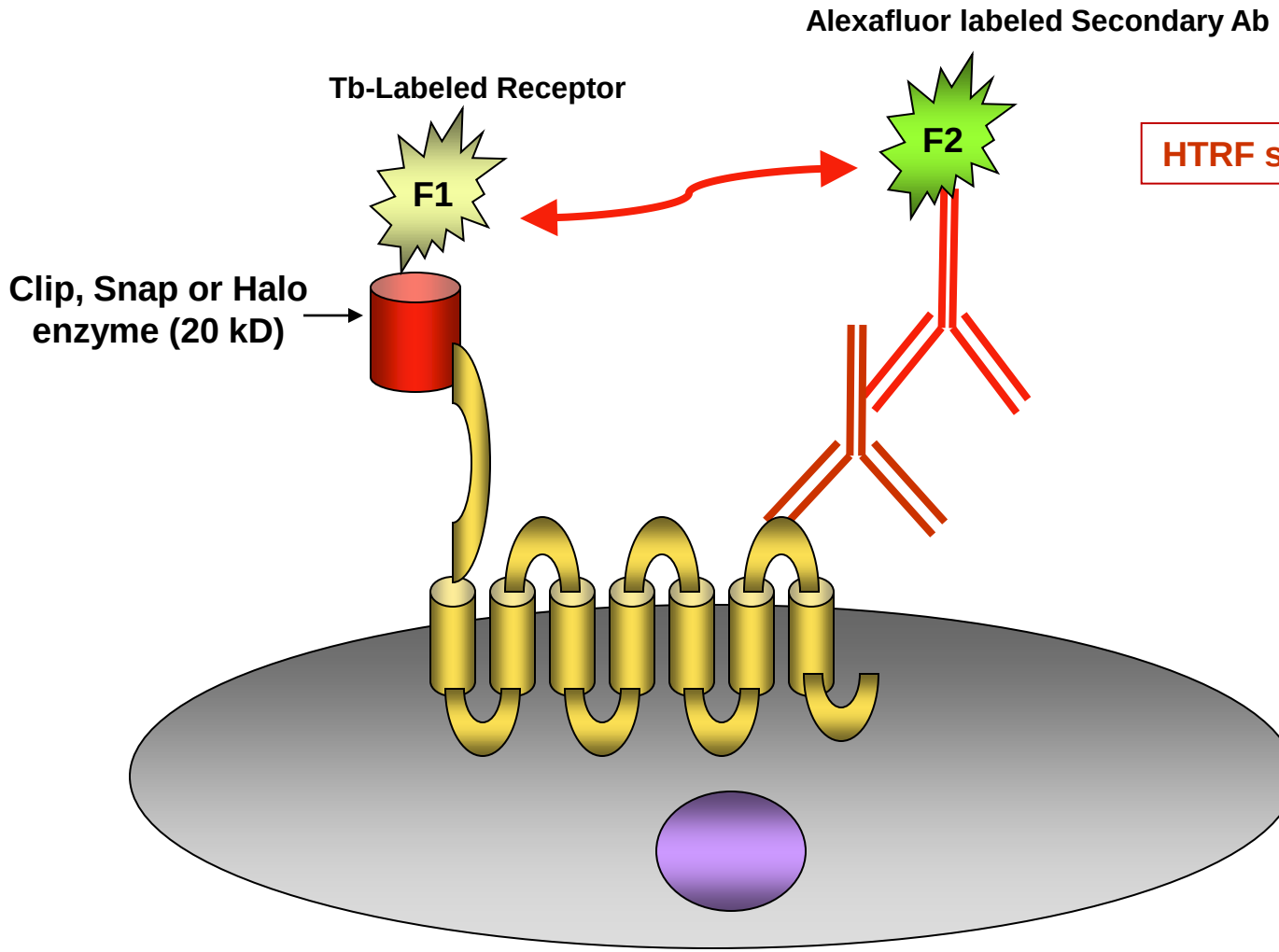
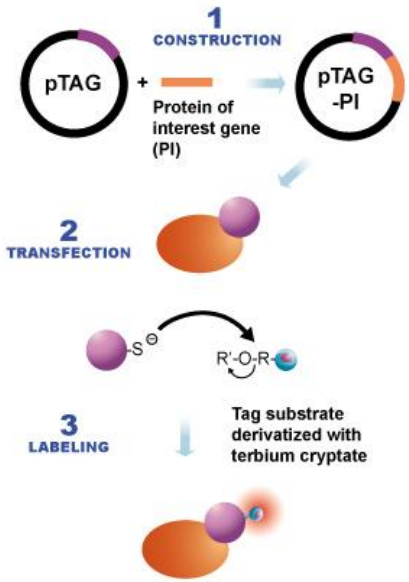
Tag-lite Technology: Applied to two GPCR Targets

Mix and Read ☺

Ligand or Ab binding to GPCR target



HTRF signal when binding occurs



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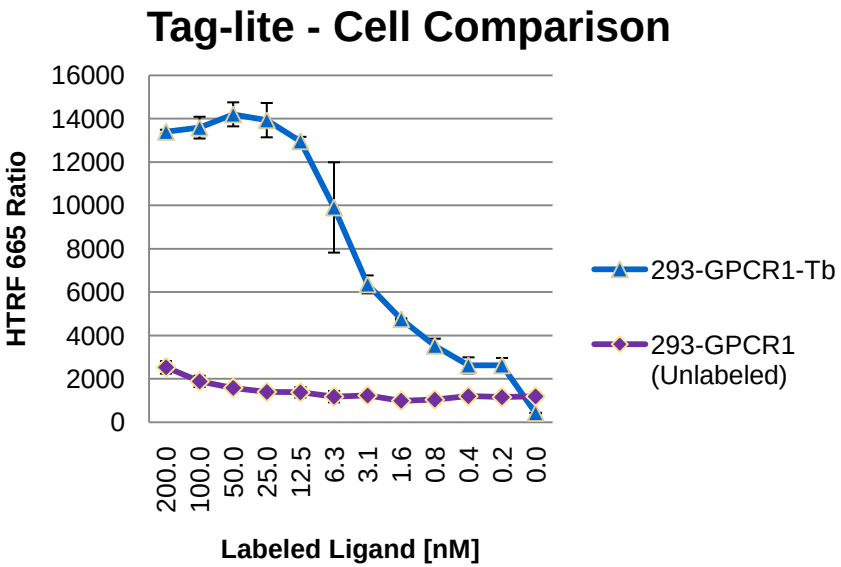
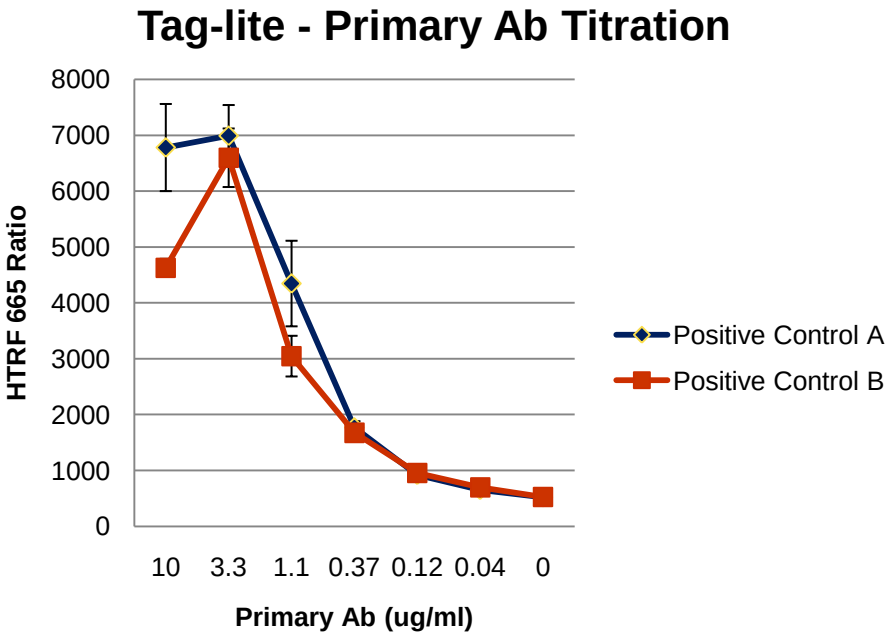
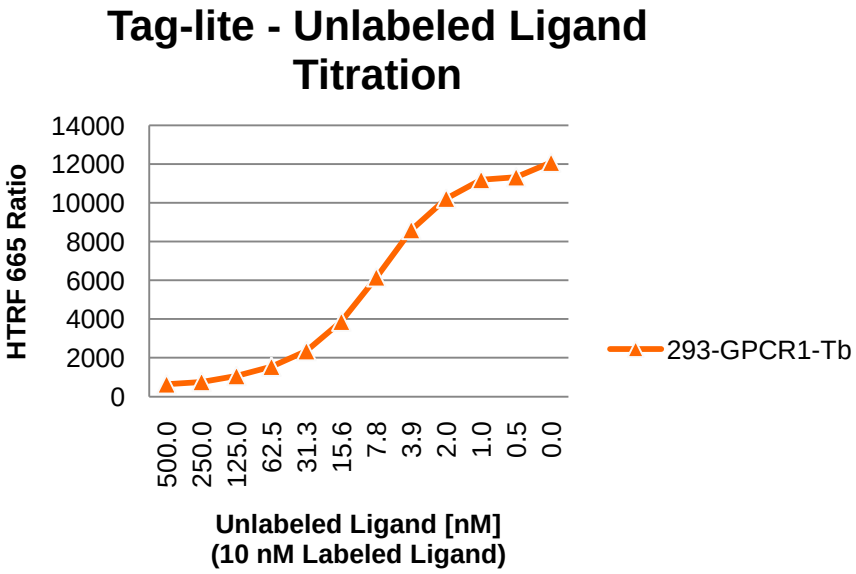
GPCR 1 – HTRF Ligand & Ab Binding to Cells







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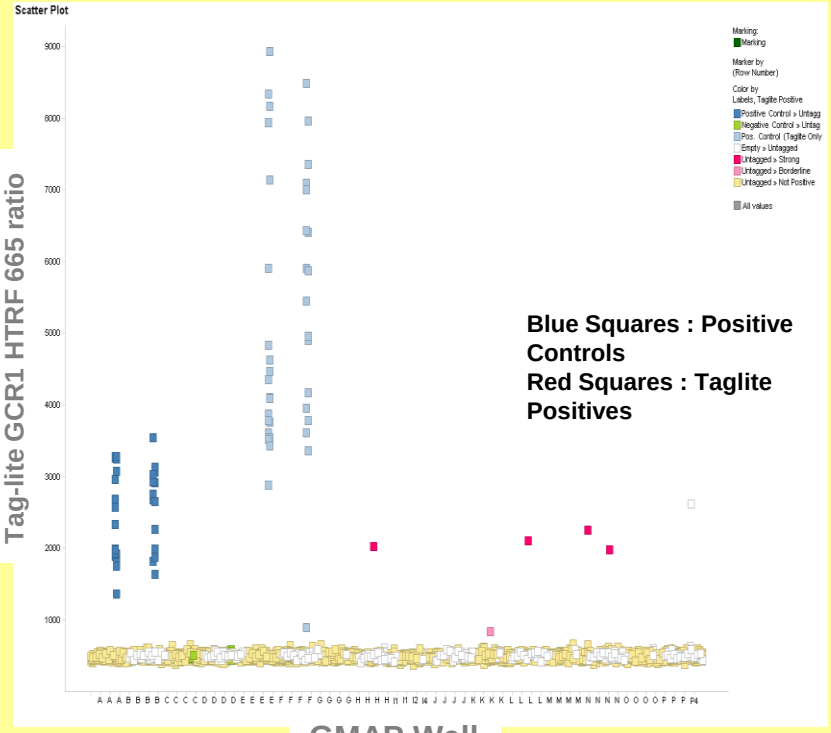
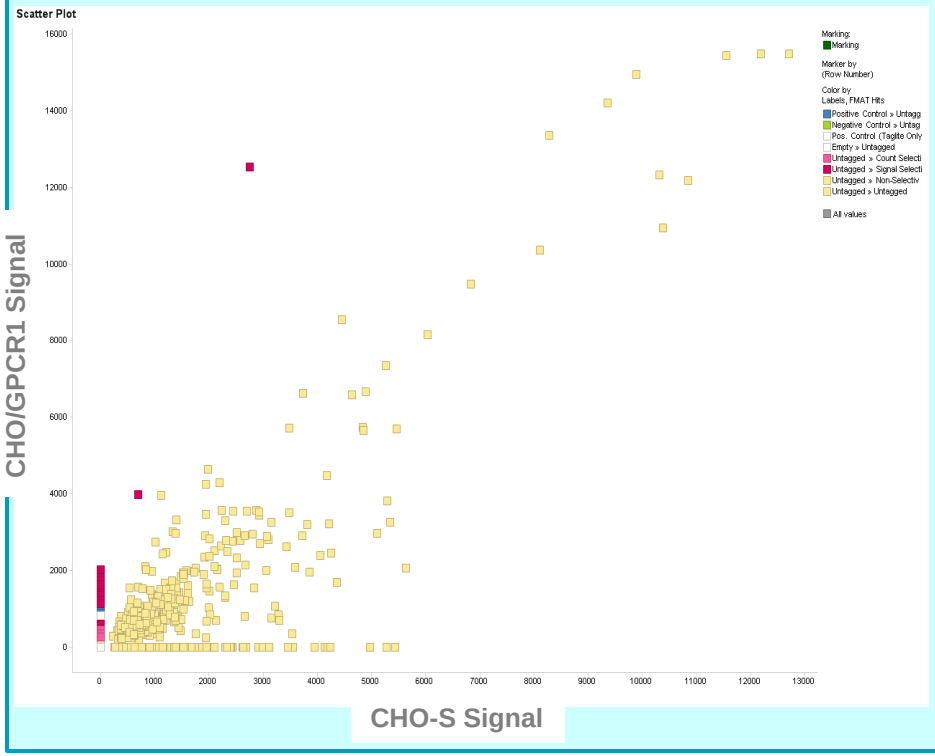
Taglite – CHO/GPCR1 – Binding of Ab supernatants

CHO/GPCR1 - Tag-lite
GPCR1-Tb



CHO-GPCR1 - FMAT



Many non-specific hits identified when FMAT was used
Results much cleaner with Tag-lite

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Ab Binding on Various Cell Lines - FMAT

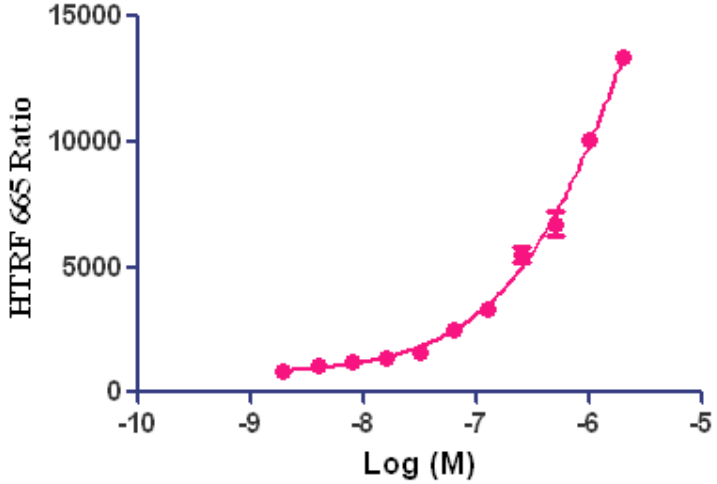
Clone	CHO-Ag	CHO	293-Ag	293	293Ag Snap	293Ag SnapTb	293-irrAg SnapTb
Pos.Control	+	-	+	-	+	+	-
MuIgG (-cont)	-	-	-	-	-	-	-
Ab Clone 1	+	+	-	-	-	-	-
Ab Clone 2	+	-	+	-	-	-	-
Ab Clone 3	-	-	+	-	+	+	-
Ab Clone 4	+	+	+	+	+	+	+

Original screening run in // by FMAT and Tag-lite

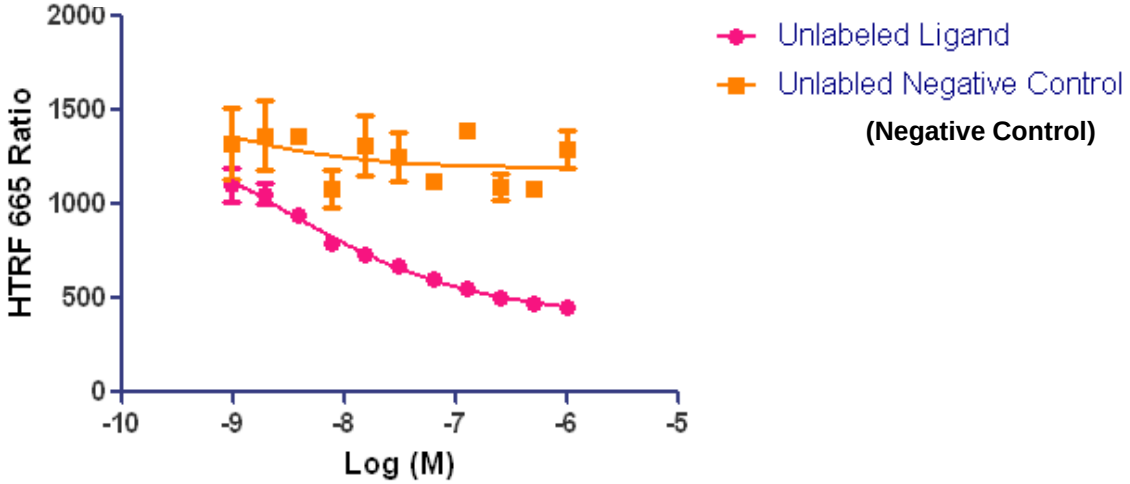
GPCR 2 - HTRF Ligand Binding

No ligand binding cell assay available due to low affinity binding of ligand to GPCR receptor
No possibility to assess blocking activity of antibodies or small molecule inhibitors

Labeled Ligand Titration



Unlabeled Ligand Competition (50 nM labeled Ligand)



50 nM ligand chosen for further inhibition experiments
(balancing the needs for maximizing the signal and minimizing ligand concentration)

Cold ligand IC50 = ~ 3nM

High concentrations of ligand are needed

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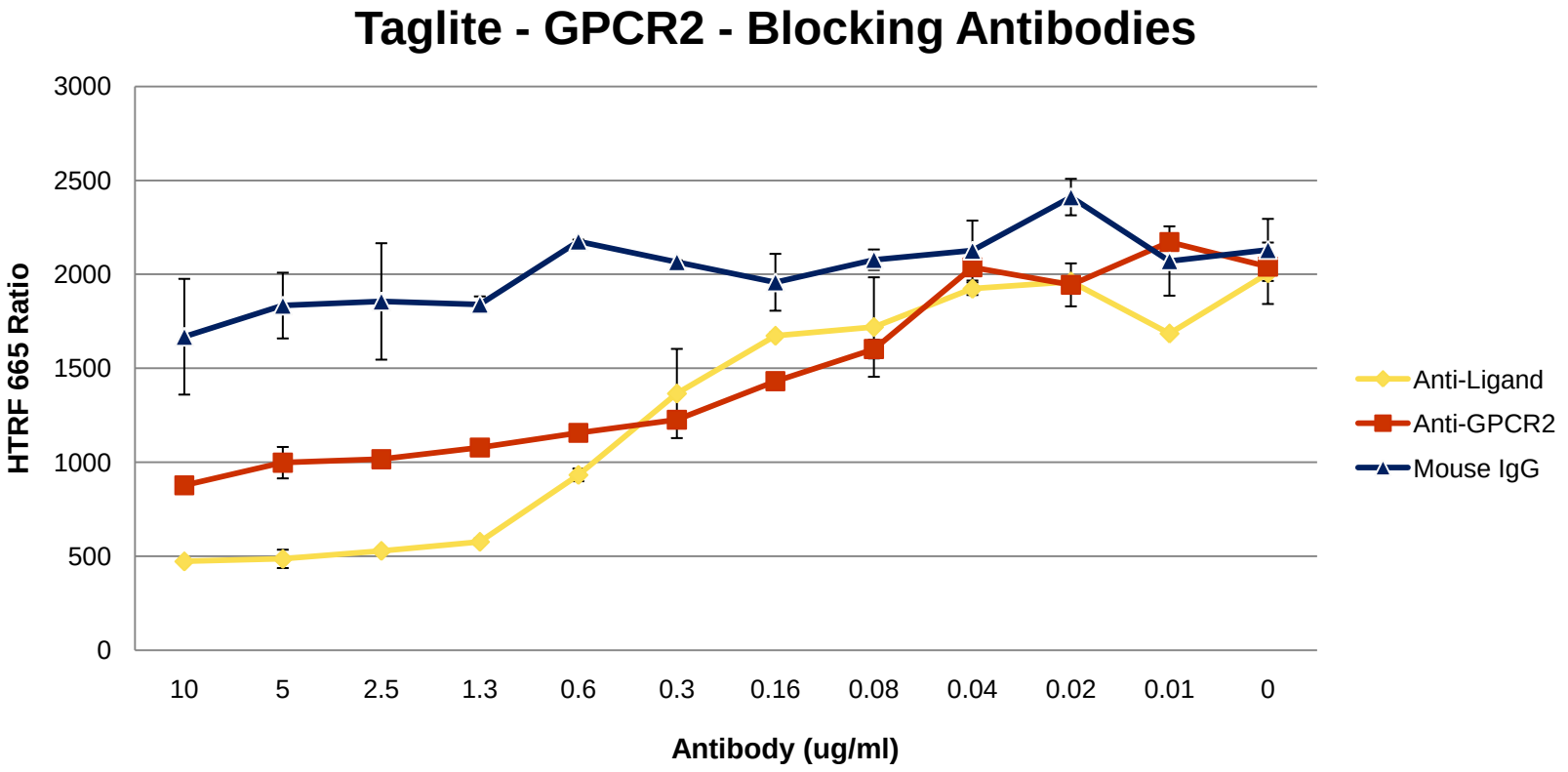
Inhibition by Blocking Antibodies



Anti-ligand Ab preincubated with ligand for 15 minutes
Anti-GPCR2 and mIgG preincubated with cells for 15 minutes

Both anti-ligand Ab and anti-GPCR2 Ab demonstrate significant inhibition of ligand binding, while irrelevant mouse IgG does not

Anti-GPCR2 does not appear to reach 100% inhibition, even when saturating (background is ~500)

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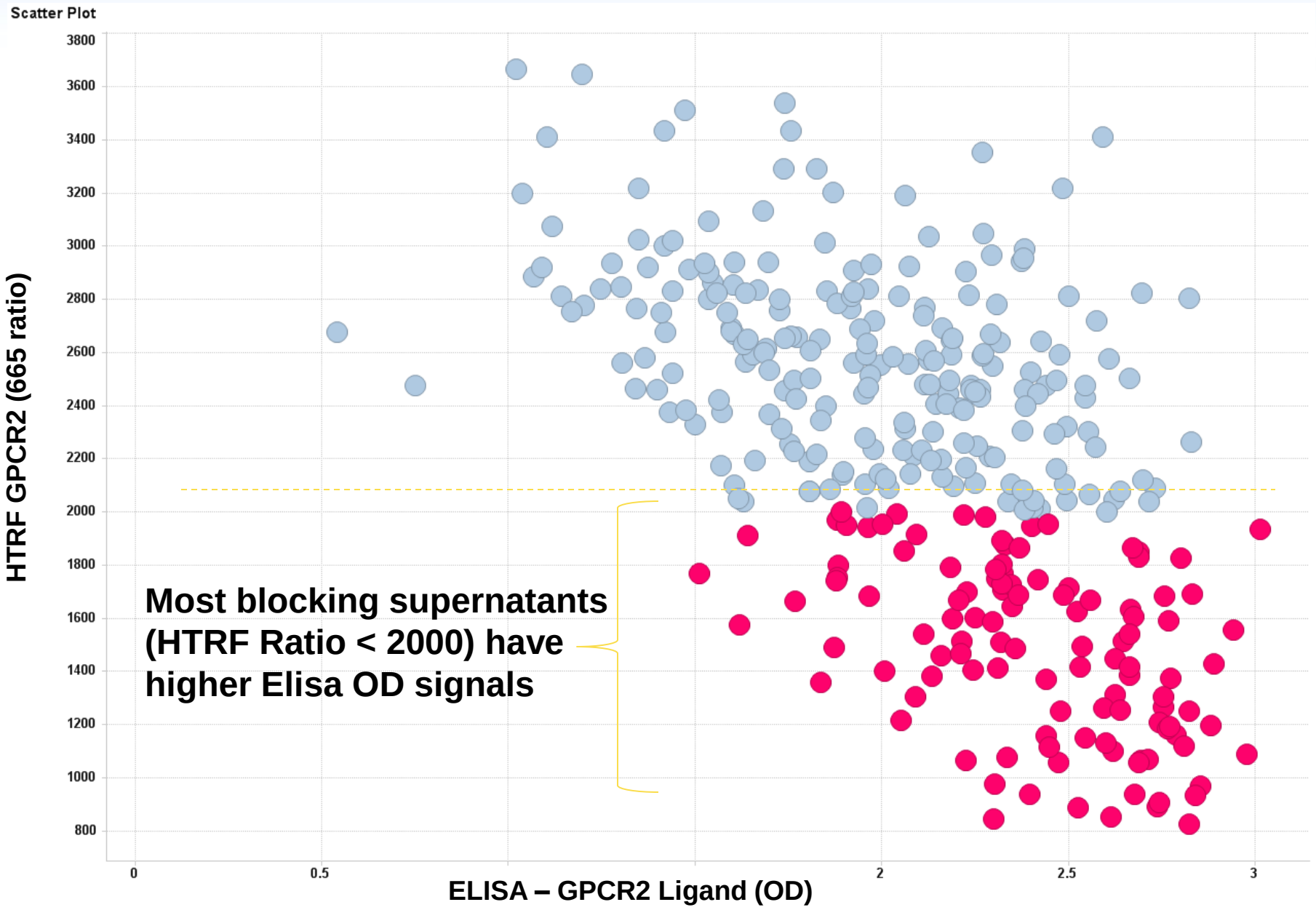
Blocking with Anti-GPCR2 Ligand Supernatants
ELISA (binding) versus Tag-lite (blocking)



Taglite Conclusions

Strengths:

- Easy, rapid, robust assay development
- Faster to execute than FMAT
- Less influenced by non-specific cell binding
- Can examine ligand/receptor interactions, even in cases where ligand binding assay could not be established using other means due to low affinity binding of ligand
- Can use same cells to look at binding and signaling

Weaknesses

- Presence of Snap tag on the receptor can affect antibody binding
- Labeling ligand can alter its properties

Thanks to

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Paula So

Andrea Tatum

Anan Chuntharapai

Helen Cai

Erika Meaddough

Peter Brams – Keith Joho – Nils Lonberg