Identification of Androgen Receptor Antagonists Using Tox21 qHTS Data and the MARCoNI Assay

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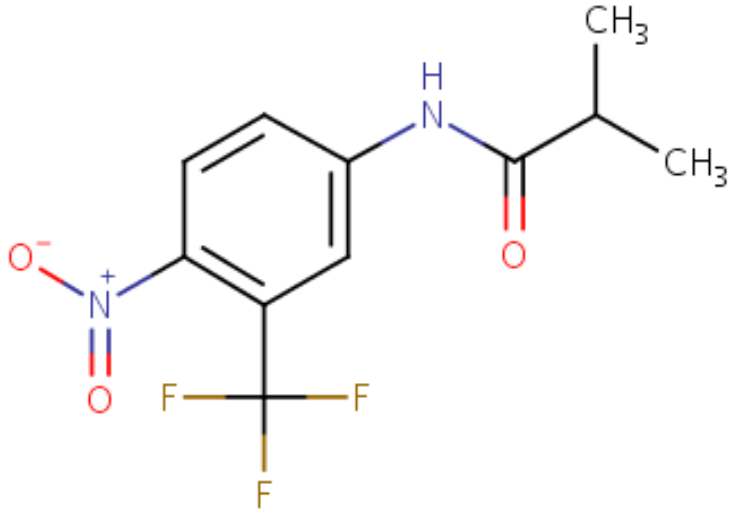
Conflict of Interest

I declare no
conflict of interest

AR Antagonism

- AR controls male sexual development and adult male sexual phenotype
- Antagonists bind to AR and inhibit receptor-mediated transcriptional activity
  - Exploited for prostate cancer drugs- flutamide, bicalutamide
- Important mechanism of endocrine disruption in environmental chemicals
  - Associated with reproductive tract and male sexual development abnormalities
  - include vinclozolin, DDE
- Need to identify other chemicals that can act as AR antagonists
- Target of EDSP and Tox21 screening efforts



Flutamide

Tox21

- Federal collaboration between NIH, EPA, FDA
- Goal is to develop methods to quickly and efficiently test chemical toxicity
- Tox21 10K chemical library qHTS data produced at NIH/NCATS
- Environmentally important chemicals
- 15 conc, n>= 3
- 1536-well plate format
- Data processed by ToxCast data pipeline (tcpl R package) to produce hit calls (active or inactive) and AC50 values

Project Goals

1) Identify AR antagonists using Tox21 qHTS AR antagonist assay data

2) Identify structural groupings of AR antagonists

3) Use MARCoNI co-regulator recruitment assay to determine patterns of co-regulator recruitment that identify true antagonists

BLA vs. LUC assay platforms in Tox21 qHTS

BLA

- Transactivation
- GAL4 β-lactamase reporter gene assay (mammalian one-hybrid)
- HEK293T human kidney cell line
- Ligand-binding domain (LBD) only
- Matching viability assay

LUC

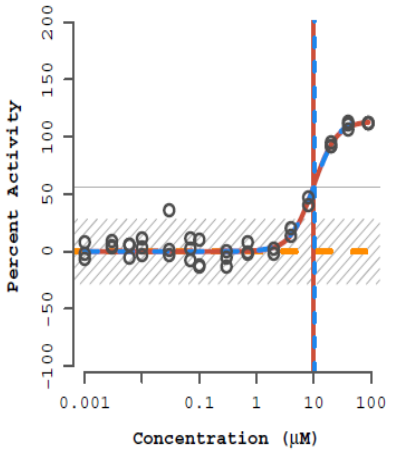
- Transactivation
- Luciferase-based reporter gene assay
- MDA-kb2 human breast cell line
- Full receptor
- Matching viability assay
- Run at second, lower agonist concentration (LUC2)

Results

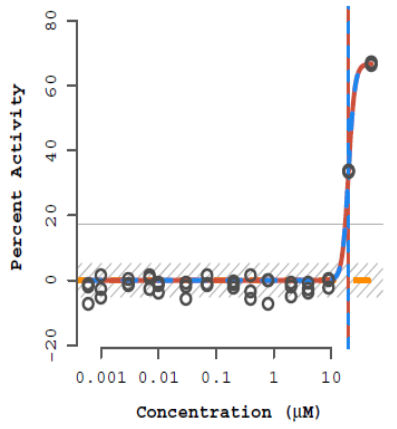
Assay	Tested	Active (% of tested)	Active <10uM (% of active)	Active <1 uM (% of active)	Active < 100 nM (% of active)
BLA	8307	1212 (15%)	382 (32%)	139 (11%)	49 (4%)
BLA VIA	8307	623 (8%)	181 (29%)	64 (10%)	20 (3%)
Both	---	575 (92% of BLAVIA)	---	---	---
LUC	8307	875 (11%)	230 (26%)	82 (9%)	18 (2%)
LUCVIA	8304	700 (8%)	107 (15%)	24(3%)	5 (0.7%)
Both		575 (82% of BLAVIA)	---	---	---
LUC2	7523	1206 (16%)	322 (27%)	103 (9%)	35 (3%)
LUC2VIA	7872	853 (11%)	137 (16%)	36 (4%)	15 (2%)
Both	---	682 (80% of BLAVIA)	---	---	--

Challenges with assessing NR antagonism *in vitro*

- Measuring loss of signal-confounded by cytotoxicity
- To address:
  - Two different assay platforms
  - Use bootstrapping techniques to determine effect of cytotoxicity
  - Two concentrations of agonist R1881
  - MARCoNI assay for corepressor/activator recruitment




```
ASSAY:   AEID1816 (TOX21_AR_LUC_MDAKB2_Antagonist2)

NAME:    Benzethonium chloride
CHID:    23810   CASRN: 121-54-0
SPID(S): Tox21_202488
M4ID:    4754104

HILL MODEL (in red):
      tp       ga       gw
val:  114      0.996    2.14
sd:   2.71     0.0262   0.208

GAIN-LOSS MODEL (in blue):
      tp       ga       gw       la       lw
val:  117      1.01     2.04     2.13     8.85
sd:   NaN      NaN      NaN      NaN      NaN

      CNST       HILL       GNLS
AIC:  441.3      291.95     295.54
PROB: 0          0.86       0.14
RMSE: 50.36      8.11       8.1

MAX_MEAN: 112      MAX_MED: 112      BMAD: 9.44

COFF: 56.6   HIT-CALL: 1   FITC: 41   ACTP: 1
```




```
ASSAY:   AEID1817 (TOX21_AR_LUC_MDAKB2_Antagonist2_viab:

NAME:    Benzethonium chloride
CHID:    23810   CASRN: 121-54-0
SPID(S): Tox21_110403_1
M4ID:    10861077

HILL MODEL (in red):
      tp       ga       gw
val:  66.8     1.3      8
sd:   1.03     0.00424  4.68

GAIN-LOSS MODEL (in blue):
      tp       ga       gw       la       lw
val:  66.8     1.3      8        3.49     6.26
sd:   NA       NA       NA       NA       NA

      CNST       HILL       GNLS
AIC:  345.27     225.7      229.7
PROB: 0          0.88       0.12
RMSE: 19.49      2.82       2.82

MAX_MEAN: 66.7      MAX_MED: 67      BMAD: 1.74

COFF: 17.4   HIT-CALL: 1   FITC: 41   ACTP: 1

FLAGS:
```

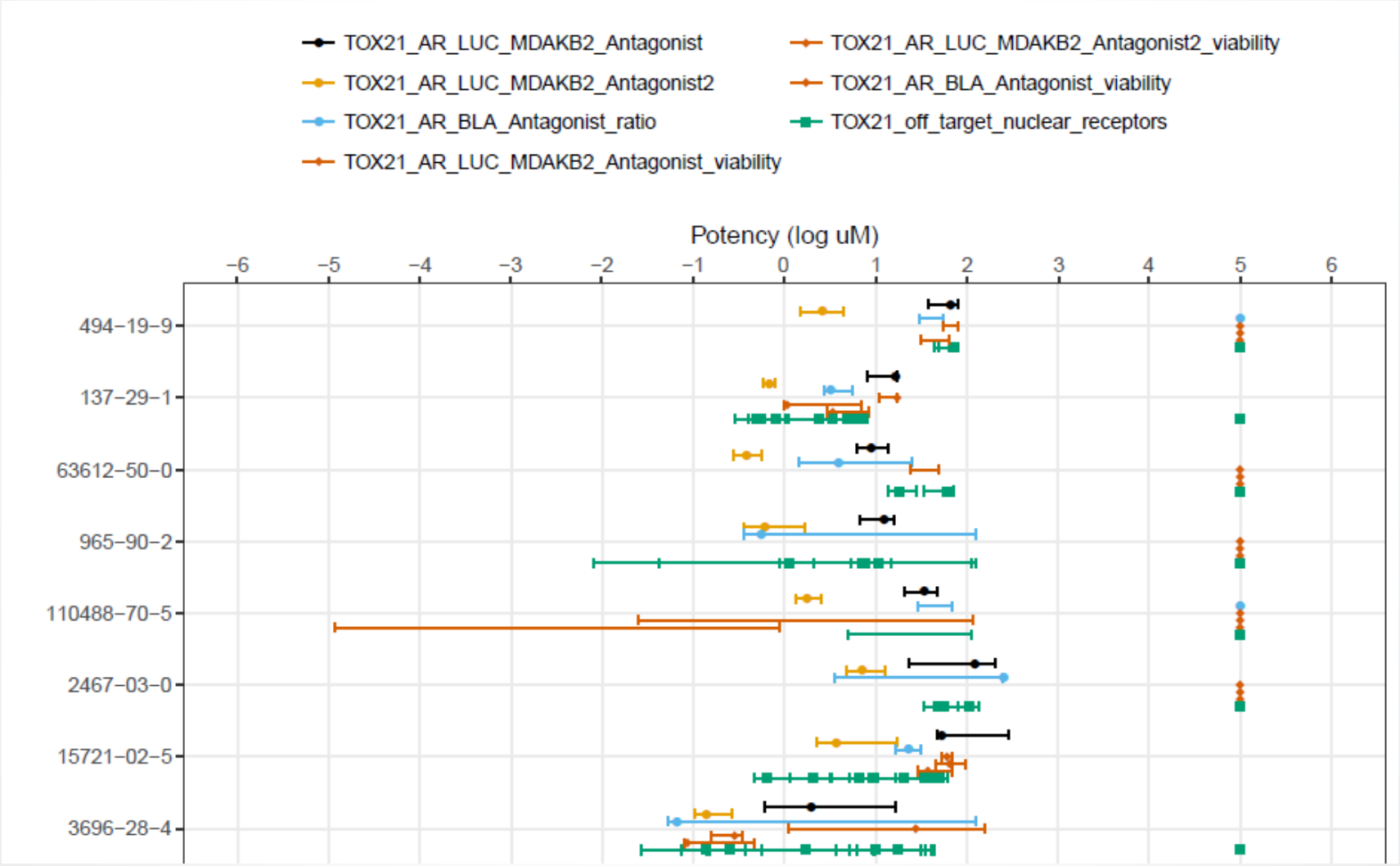
Objective 1

Objective 1: Use bootstrapping of response curve fitting to assess criteria for true antagonism

Why use bootstrapping?

- Comparing AC50s
  - AR assays to their matching viability assays for cytotoxicity
  - LUC (high agonist concentration) to LUC2 (low agonist concentration) for shift
- Comparing AC50s directly does not take into account model variability
- Use bootstrapping methods to produce 95% confidence intervals around the AC50 values
  - Statistical methods that uses resampling methods to produce confidence intervals
  - Removes one data point from population and re-runs model 1000X to give distribution of values for AC50
  - Not related to biological variability which is accounted for in experimental design
  - R package “toxboot” by Eric Watt

Bootstrapping results



Reference Chemical Results

Chemical	Designation	Assay Hitcalls	LUC vs. LUC2	LUC2 vs. LUC2VIA
Procymidone	Very Weak Antagonist	BLA, LUC, LUC2	Yes	Yes
Fenarimol	Very Weak Antagonist	BLA, LUC, LUC2	Yes	Yes
4-(1,1,3,3-Tetramethylbutyl)phenol	Weak Antagonist	LUC2	Yes	Yes
o,p'-DDT	Weak Antagonist	BLA, LUC2	Yes	Yes
p,p'-DDE	Weak Antagonist	LUC2	Yes	Yes
Propiconazole	Weak Antagonist	BLA, LUC, LUC2	Yes	No
Zearalenone	Weak Antagonist	BLA, LUC, LUC2	No	No
Methoxychlor	Weak Antagonist	BLA, LUC, LUC2	No	No
Linuron	Moderate/Weak Antagonist	BLA, LUC2	Yes	No
Vinclozolin	Moderate/Weak Antagonist	BLA, LUC, LUC2	Yes	Yes
Flutamide	Moderate/Weak Antagonist	BLA, LUC, LUC2	Yes	Yes
Bisphenol A	Moderate/Weak Antagonist	BLA, LUC, LUC2	Yes	Yes
Prochloraz	Moderate/Weak Antagonist	BLA, LUC, LUC2	Yes	Yes
Cyproterone acetate	Moderate Antagonist	BLA, LUC	Yes	Yes
Nilutamide	Moderate Antagonist	BLA, LUC, LUC2	Yes	Yes
Spironolactone	Strong/Moderate Antagonist	BLA, LUC	No	Yes
Mifepristone	Strong/Moderate Antagonist	BLA, LUC, LUC2	No	Yes
Fenitrothion	Strong Antagonist	BLA, LUC, LUC2	Yes	Yes
Hydroxyflutamide	Strong Antagonist	BLA, LUC, LUC2	Yes	Yes
Bicalutamide	Strong Antagonist	BLA, LUC, LUC2	Yes	Yes
17-Methyltestosterone	Negative Antagonist	NA	NA	NA
4-Androstene-3,17-dione	Negative Antagonist	NA	NA	No
Atrazine	Negative Antagonist	NA	NA	NA
Daidzein	Negative Antagonist	BLA	NA	NA
Deltamethrin	Negative Antagonist	NA	NA	NA
Methomyl	Negative Antagonist	LUC2	NA	No
Simazine	Negative Antagonist	NA	NA	NA
Testosterone	Negative Antagonist	NA	NA	NA

Sensitivity/Specificity Criteria

Sensitivity= # of true positives/(# of true positives + # of false negatives)
Specificity= # of true negatives/(# of true negatives + # of false positives)

Criteria	Sensitivity	Specificity	Balanced Accuracy
Active in BLA	0.9	0.88	0.89
Active in LUC	0.8	1	0.9
Active in LUC2	0.9	0.88	0.89
Active in at least 2 assays	0.9	1	0.95
Active in all three assays	0.7	1	0.85
Active in all three assays, LUC vs. LUC2 difference, not confounded by cytotoxicity	0.5	1	0.75
Active in LUC2, LUC vs. LUC2 difference, not confounded by cytotoxicity	0.7	1	0.85

Results

- Only 102 chemicals positive using strictest criteria
- Expanding criteria allows for ranking of chemicals based on strength of evidence
- Chemicals that are confounded by cytotoxicity are not eliminated but evidence is weaker
- Potency not currently considered but is another important factor

Hydroxyflutamide
Bis(tributyltin)oxide
Dipyrithione
Ziram
NTP Mix21 AR2 2-EQP
17alpha-Ethinylestradiol
Bis(1-piperidinylthioxomethyl)hexasulfide
Triphenyltin acetate
Tributyltin benzoate
Nilutamide
Triethyltin bromide
Equilin
17alpha-Estradiol
(Acryloyloxy)(tributyl)stannane
Triphenyltin fluoride
Ethylestrenol
Copper dimethyldithiocarbamate
Vinclozolin

Objective 2: Structural categorization

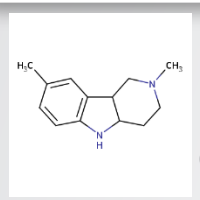
Methods for structural analysis

- Toxprints are chemical structure "fingerprints" that can be used for structural categorization of chemicals
- 729 descriptors

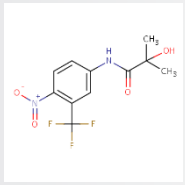
Chemical Name	bond:C(=O)O_carboxylicAcid_generic	bond:C(=O)O_carboxylic Ester_4-nitrophenol	bond:C(=O)O_carboxylic Ester_acyclic
Sodium L-ascorbate	0	0	0
L-Ascorbic acid	0	0	0
Aspartame	1	0	1
Aspirin	1	0	0
Astemizole	0	0	0
Atrazine	0	0	0
Atropine	0	0	0
Auramine hydrochloride	0	0	0
Auranofin	0	0	1
5-Azacytidine	0	0	0
6-Azacytidine	0	0	0
Azaserine	1	0	1

- Self-organizing maps (SOMs)
  - Unsupervised clustering technique
  - Clusters data into "honeycomb" like map where each cluster is surrounded by similar clusters using Tanimoto distance measure
  - Produced SOM using toxprint chemical descriptors for 8416 chemicals
  - Overlaid clusters with median of the differences between the LUC and LUC2 assay CI's

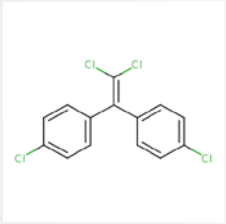
Structural SOM



(5) 787-dicarbine, tinoridine (topisomerase inhibitors)



(12) 510-Flutamides

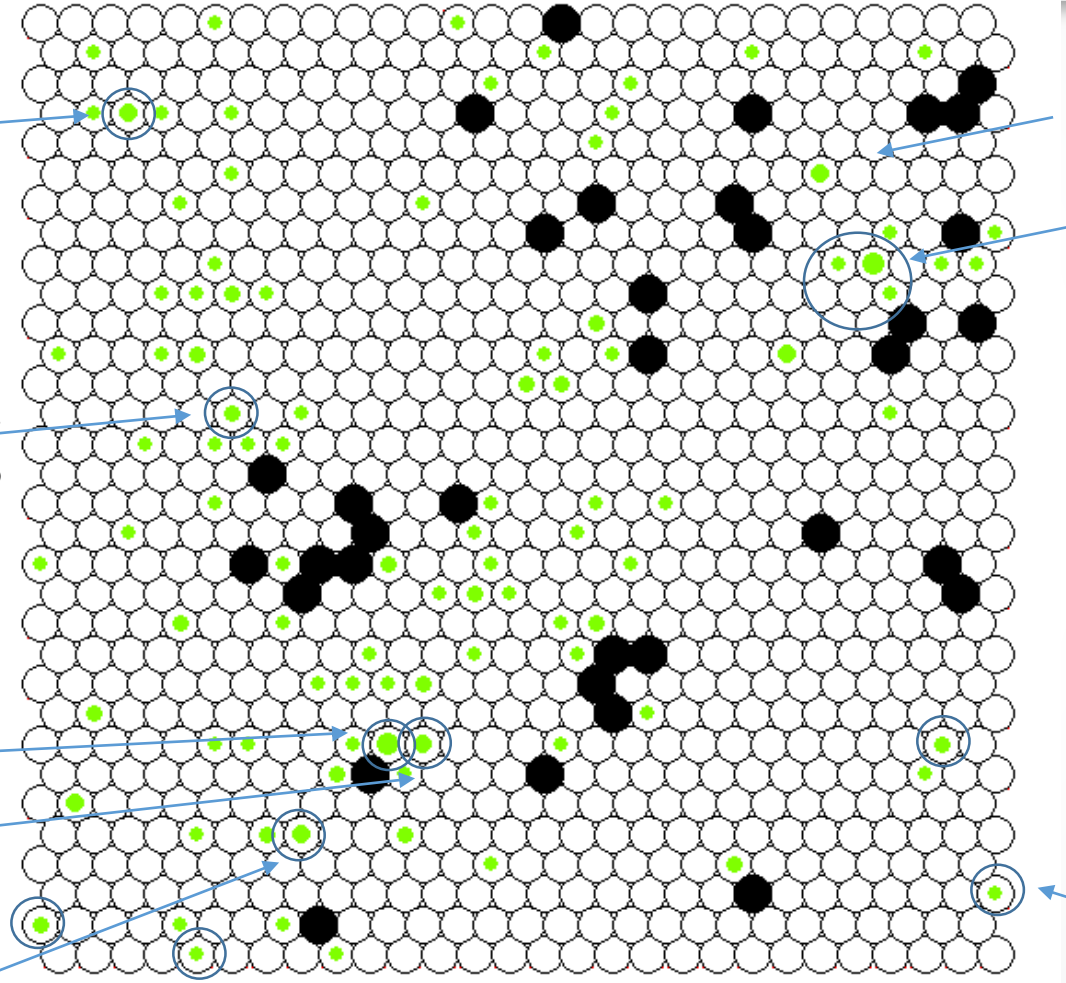


(1) 207-chlorinated biphenyls including dihydroxymethoxychlor olefin, p,p'- DDE

(3) 208-hydroxyfluorenes (known estrogenic compounds)

120-phytoestrogens, salicylates (6)

869-896
841-868
813-840
785-812
757-784
729-756
701-728
673-700
645-672
617-644
589-616
561-588
533-560
505-532
477-504
449-476
421-448
393-420
365-392
337-364
309-336
281-308
226-280
225-252
197-224
169-196
141-168
113-140
85-112
57-84
29-56
1-28



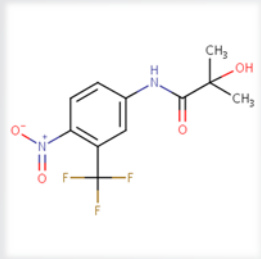
(4) 751-Guggulsterones

(2) 669-equilin/estrone/estropipate

(9) Organotins

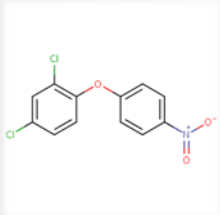
(43) Quarternary ammonium compounds

Will be replaced with new one

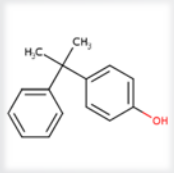


510-Flutamides (1)

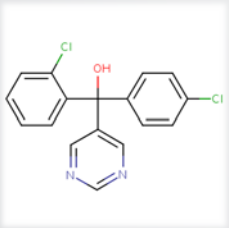
313-Azole compounds (7)



316-Chlorobenzenes with Ether linkage (6)



36-Bisphenols (10)



98-Fenarimol-like fungicides (5)

869-896
841-868
813-840
785-812
757-784
729-756
701-728
673-700
645-672
617-644
589-616
561-588
533-560
505-532
477-504
449-476
421-448
393-420
365-392
337-364
309-336
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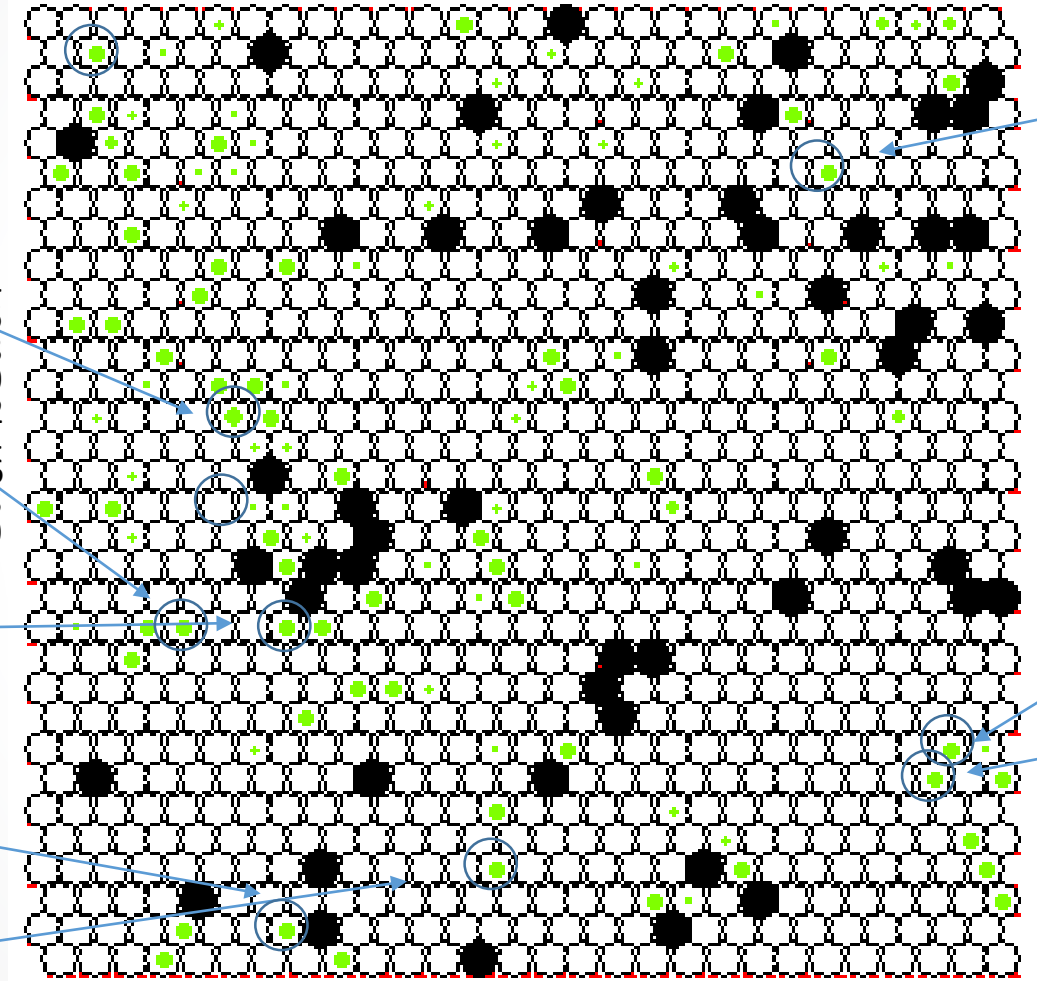
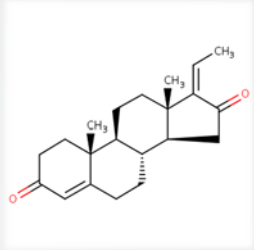
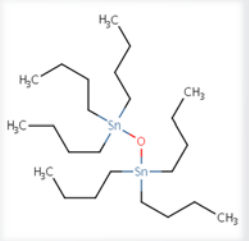
141-168
113-140
85-112
57-84
29-56
1-28



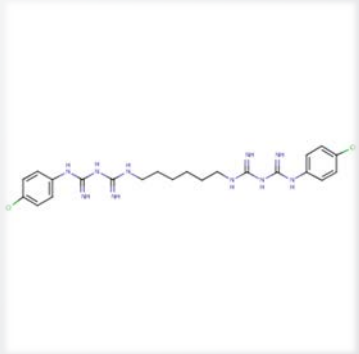
751-Guggulsterones (2)



223-Organotins (11)



194-Clorhexidine-like (3)



Objective 3: MARCoNI Assay

MARCoNI assay

- Microarray Assay for Real-time Coregulator-Nuclear receptor Interaction
- Cell-free assay measuring co-regulator recruitment to AR-LBD
  - 154 co-regulators
  - 3 concentrations (1, 10, 100 uM)
  - log fold-change of binding compared to DMSO
- Tested 318 suspected AR antagonists
- Goal of this assay is to see if patterns of coregulatory recruitment can distinguish between true antagonists and false antagonists (cytotoxicity/artifacts)

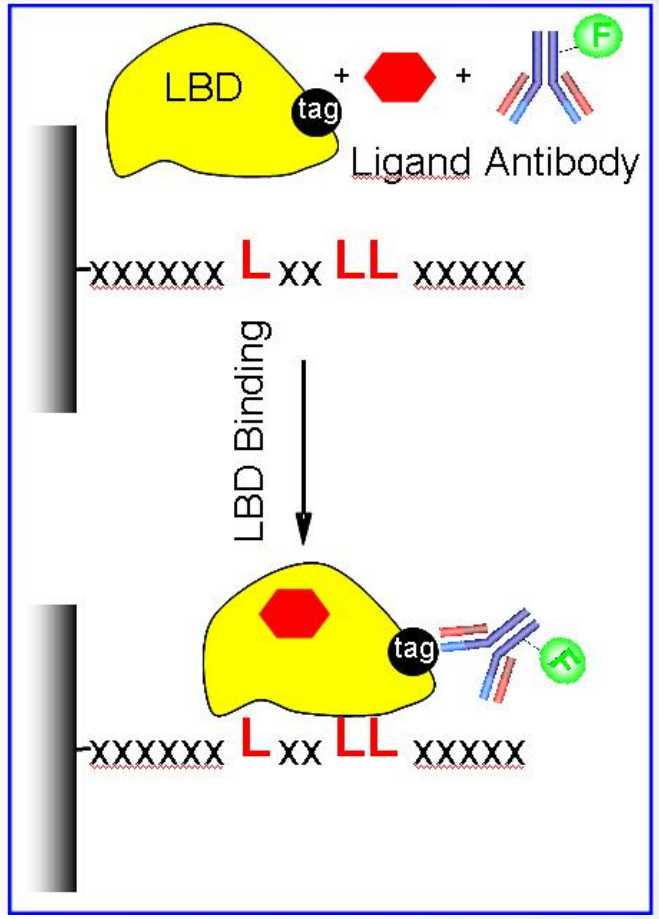


Image: pamgene.com

MARCoNI assay

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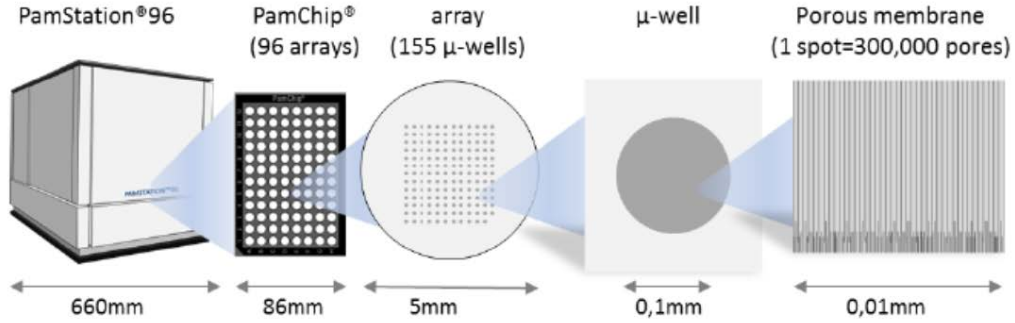


Figure 1: Outline of PamStation®96 instrument and PamChip®96 microarray, each array consisting of 155 µ-wells, (The PamChip®96 is therefore a 14880 µ-well plate)

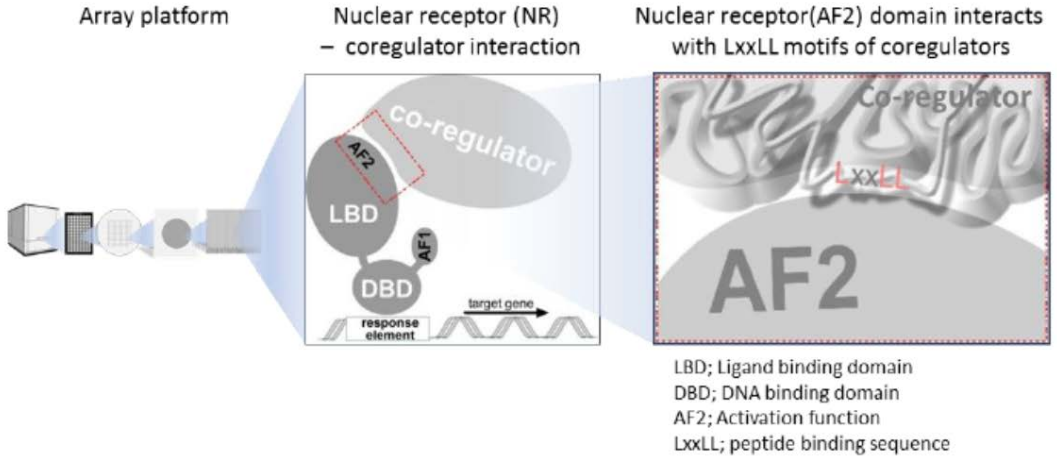
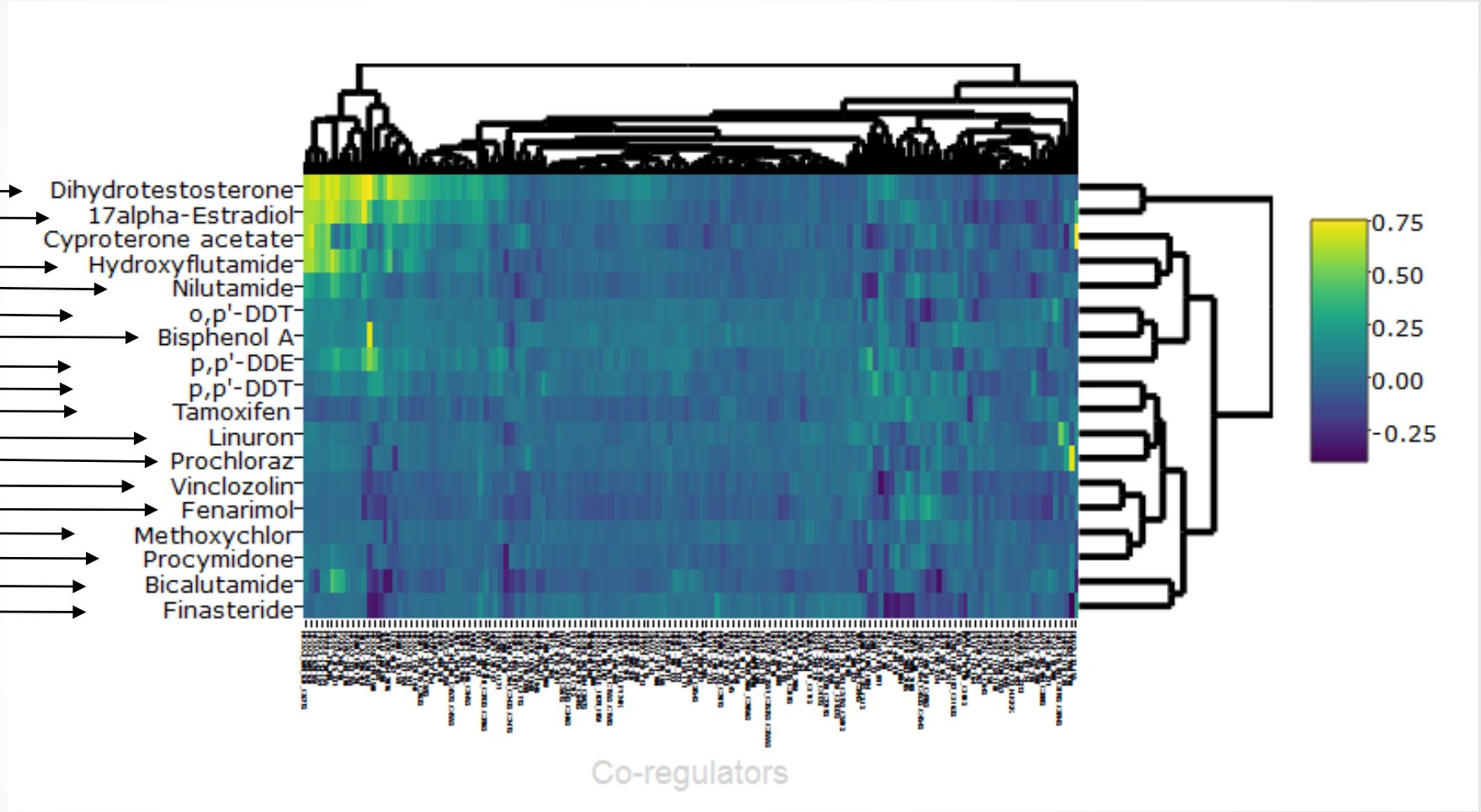


Figure 2: Outline of the nuclear receptor – coregulator interactions in the µ-wells

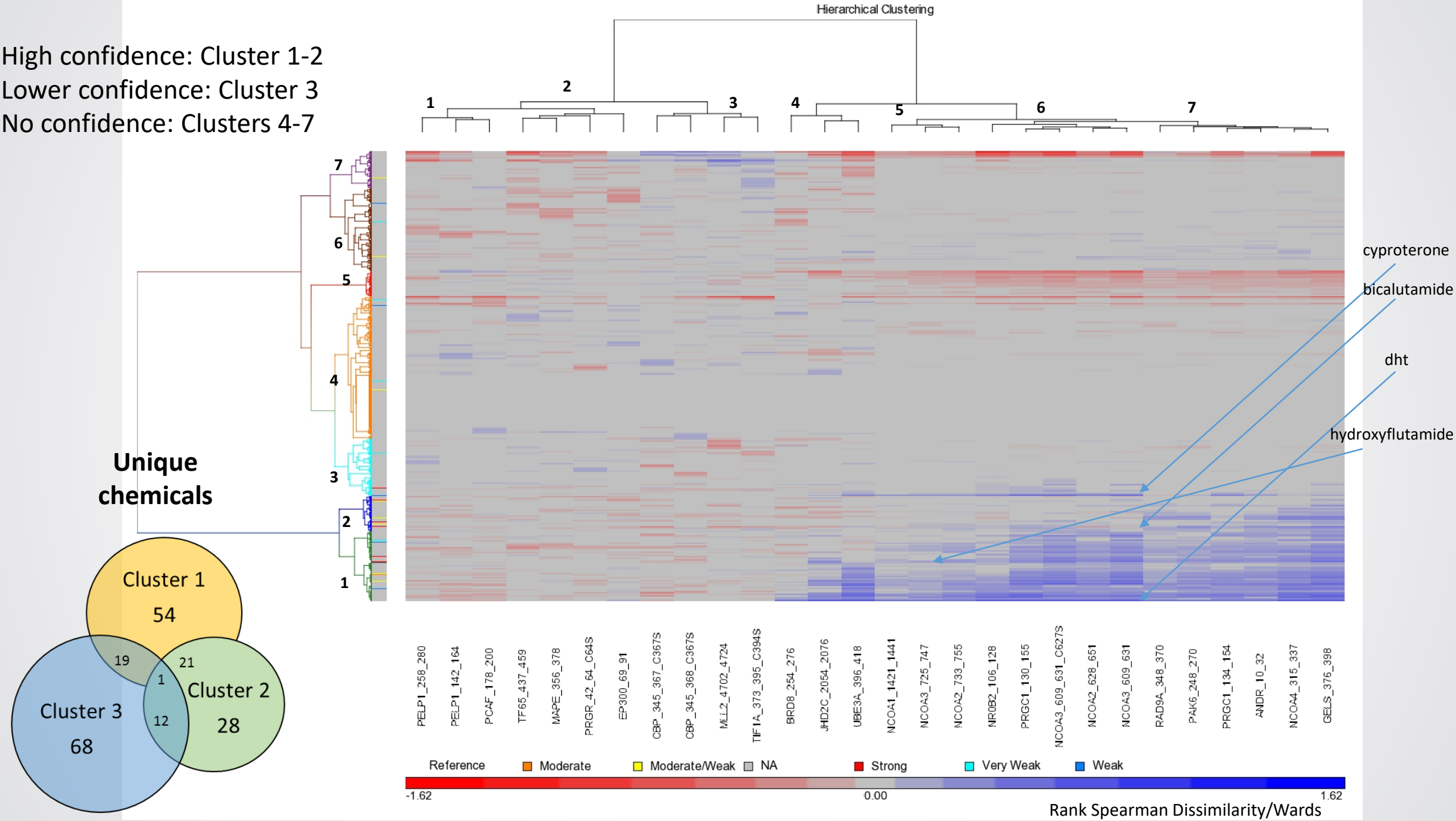
Image: pamgene.com

Keith's heatmap here

AR Activity:

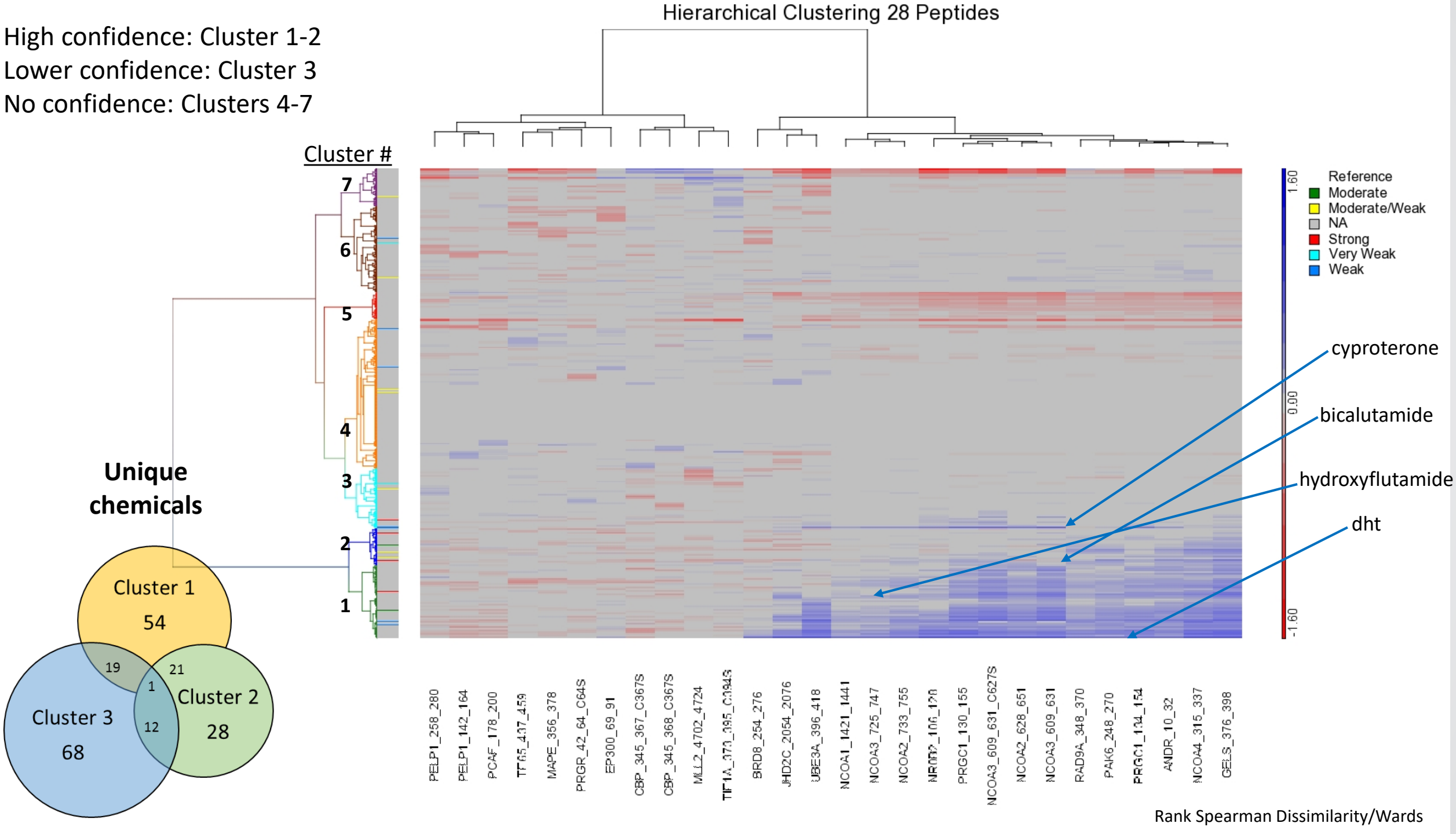


High confidence: Cluster 1-2
Lower confidence: Cluster 3
No confidence: Clusters 4-7



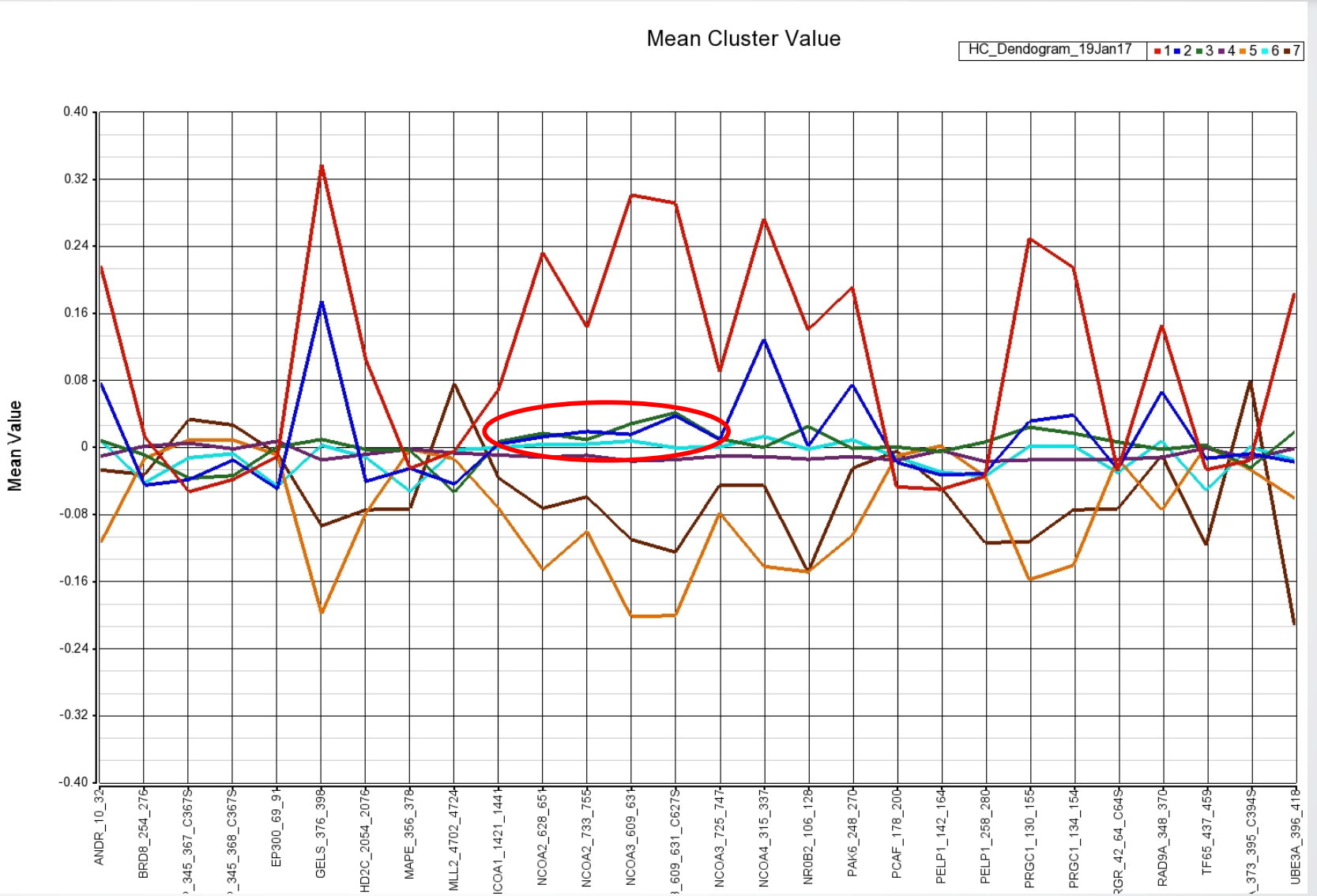
Unique chemicals

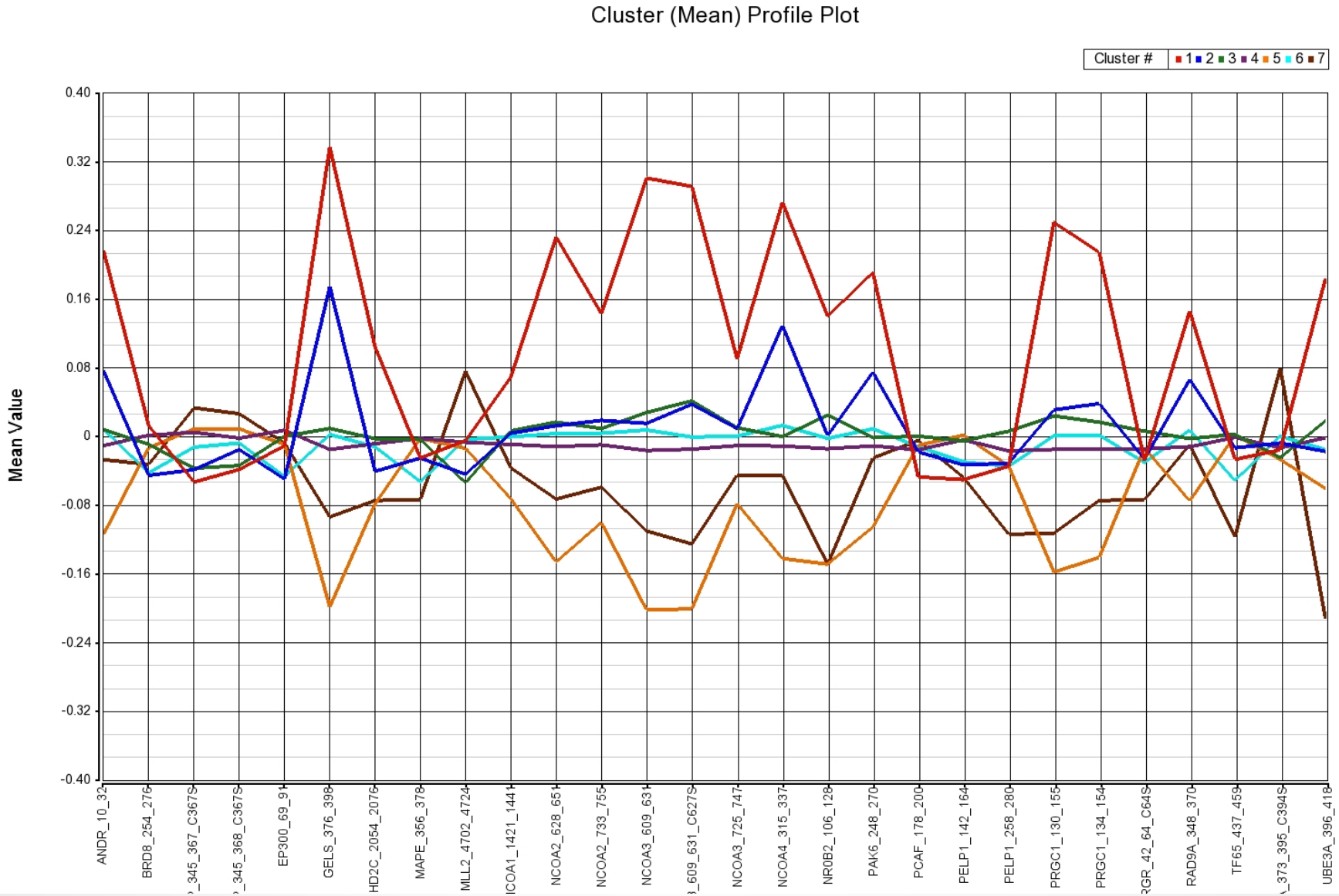
High confidence: Cluster 1-2
Lower confidence: Cluster 3
No confidence: Clusters 4-7

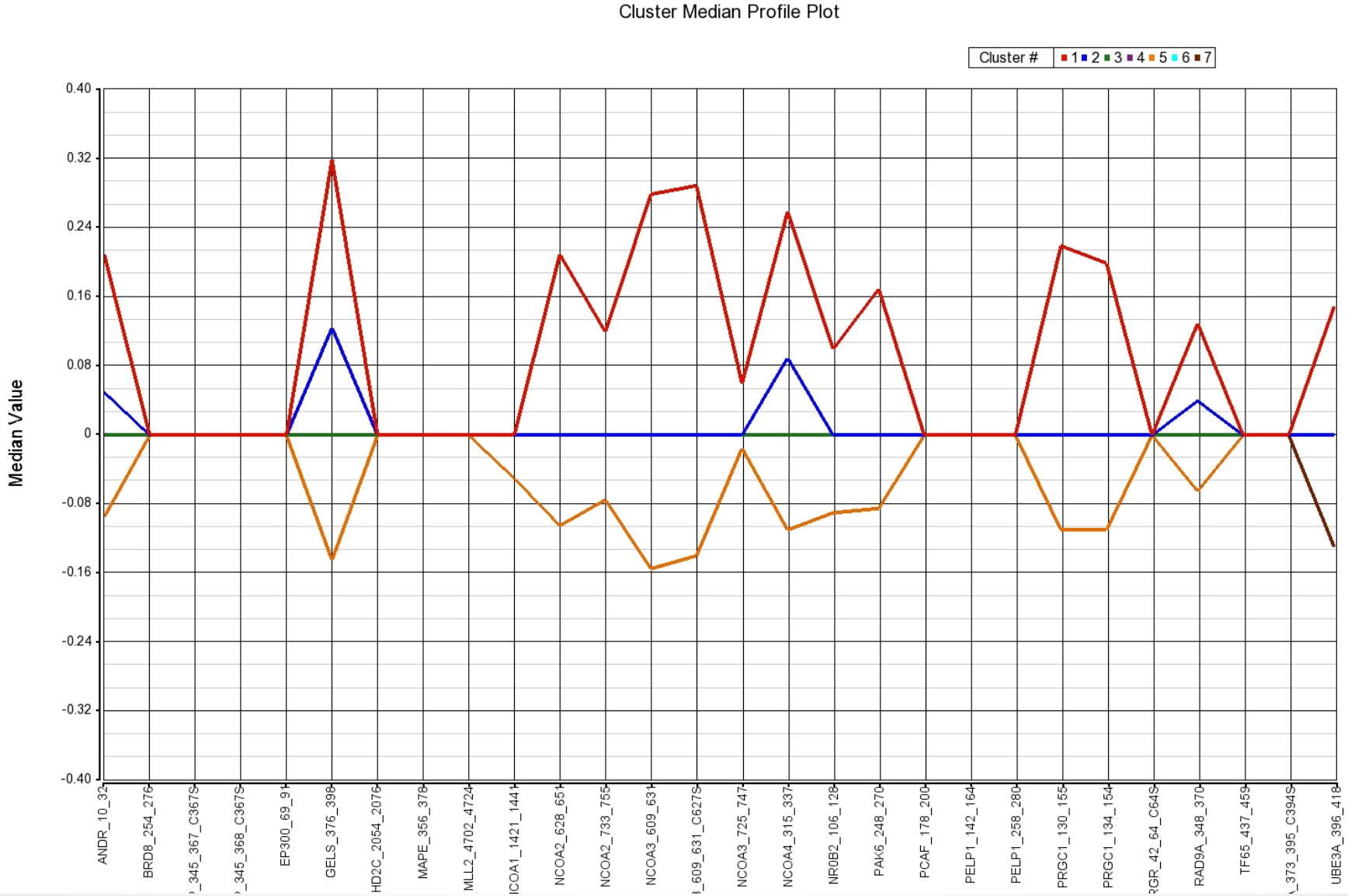


Co-regulator Recruitment Patterns

Plotted mean value for each cluster. Comparing cluster 1 vs 2, note loss of binding of a series of peptides (circled in red). All are SRC coactivators that have histone acetyl transferase activity.




Cluster (Mean) Profile Plot
Cluster # 1 2 3 4 5 6 7
Mean Value
0.40
0.32
0.24
0.16
0.08
0
-0.08
-0.16
-0.24
-0.32
-0.40
ANDR_10_32
BRD8_254_276
_345_367_C367S
_345_368_C367S
EP300_69_91
GELS_376_398
HD2C_2054_2076
MAPE_356_378
MLL2_4702_4724
COA1_1421_1441
NCOA2_628_651
NCOA2_733_755
NCOA3_609_631
_609_631_C627S
NCOA3_725_747
NCOA4_315_337
NR0B2_106_128
PAK6_248_270
PCAF_178_200
PELP1_142_164
PELP1_258_280
PRGC1_130_155
PRGC1_134_154
GR_42_64_C64S
RAD9A_348_370
TF65_437_459
_373_395_C394S
UBE3A_396_418


Cluster Median Profile Plot
Cluster # 1 2 3 4 5 6 7
Median Value
0.40
0.32
0.24
0.16
0.08
0
-0.08
-0.16
-0.24
-0.32
-0.40
ANDR_10_32
BRD8_254_276
EP300_69_9
GELS_376_398
HD2C_2054_2076
MAPE_356_378
MLL2_4702_4724
NCOA2_628_65
NCOA2_733_755
NCOA3_609_63
NCOA3_725_747
NCOA4_315_337
NR0B2_106_128
PAK6_248_270
PCAF_178_200
PELP1_142_164
PELP1_258_280
PRGC1_130_155
PRGC1_134_154
RAD9A_348_370
TF65_437_459
UBE3A_396_418

Conclusions

- Of ~8000 tested chemicals, 102 exhibited the strongest evidence for true AR antagonism.
- Different criteria can be used to optimize sensitivity and specificity based on specific goals and needs.
- X, Y, and Z structural groups showed a strong association with AR antagonism. THIS WILL BE FIXED BASED ON NEW MAP
- MARCoNI assay identified multiple patterns of co-regulator recruitment associated with several categories of true and false positives.
- Patterns of co-regulator recruitment can be investigated for specific adverse effects
- This data can be used to rank chemicals that are deserving of prioritization for follow-up testing.

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Questions?

Questions or comments?

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