Getting to recombinant antibodies that guarantee reproducible research

Andrew Bradbury, Specifica Inc

Why move towards recombinant Abs?

- Many commercial reagent antibodies have problems
  - Both polyclonals and monoclonals
- Monoclonals antibodies may not be monoclonal
  - Recombinants from monoclonals perform better than original monoclonals, even without additional chains
- Sequenced recombinant antibodies are never lost –
  - They can always be re-synthesized and re-expressed
  - This ensures reproducibility
    - (differences between batches of a recombinant are far less than between batches of polyclonals)
- Recombinants need be characterized only once –
  - But different lots always need to have binding activity confirmed
- Recombinant antibodies are highly versatile reagents (e.g. in fusions etc.)

Starting with scary stories: clinical trials with an ERCC1 "Biomarker"

- Low levels of ERCC1 (mAb 8F1 detection) predicted efficacy of cisplatin-based adjuvant chemotherapy in non-small cell lung cancer

But:

- Manufacturer changed something in the mAb 8F1 reagent

So:

  - Tumors previously "low ERCC1" showed "high ERCC1" immunostaining
  - Now high ERCC1 staining was *no longer predictive of efficacy*
- mAb 8F1 also discovered to bind CCTa
  - Recognition of CCTa, not ERCC1, caused dominant mAb 8F1-immunoreactivity in a subset of mAb 8F1-positive cancers
  - **High CCTa expression**, not ERCC1, predicted longer disease-free and overall survival in some lung cancers

Vaezi et al., Cancer 120: 1898-1907 (2014).

Antibodies against estrogen receptor ß

- 12 of 13 antibodies (including the most commonly used) showed non-specific binding
  - The only specific antibody was rarely used
  - With this reagent: ERß protein levels correlate with ERß mRNA levels
    - In : testis, ovary, placenta (weakly), lymphoid cells, granulosa cell tumors, subset of malignant melanoma and thyroid cancers
- ERß expression commonly reported in breast and breast cancer
  - Many publications
  - 20 years of cancer therapy projects based on antibody-identified ERß expression in breast cancer
    - **But: There is no ERß mRNA expressed in breast or breast cancer!**
  - Self reinforcing dogma

Andersson, S. *et al.*, *Nat Commun* **8**, 15840, (2017).

Antibody validation

Jennifer Bordeaux, Allison W. Welsh, Seema Agarwal, Elizabeth Killiam, Maria T. Baquero, Jason A. Hanna, Valsamo K. Anagnostou, and David L. Rimm
Department of Pathology, Yale University School of Medicine, New Haven, CT, USA

BioTechniques 48:197-209 (March 2010) doi 10.2144/000113382
Keywords: antibody; validation; immunohistochemistry; immunofluorescence

Antibodies are among the most frequently used tools in basic science research and in clinical assays, but there are no universally accepted guidelines or standardized methods for determining the validity of these reagents. Furthermore, for commercially available antibodies, it is clear that what is on the label does not necessarily correspond to what is in the tube. To validate an antibody, it must be shown to be specific, selective, and reproducible in the context for which it is to be used. In this review, we highlight the common pitfalls when working with antibodies, common practices for validating antibodies, and levels of commercial antibody validation for seven vendors. Finally, we share our algorithm for antibody validation for immunohistochemistry and quantitative immunofluorescence.

An Open Letter to Our Readers on the Use of Antibodies

Blind faith that the antibody will stain whatever the manufacturer claims is not consistent with good science.

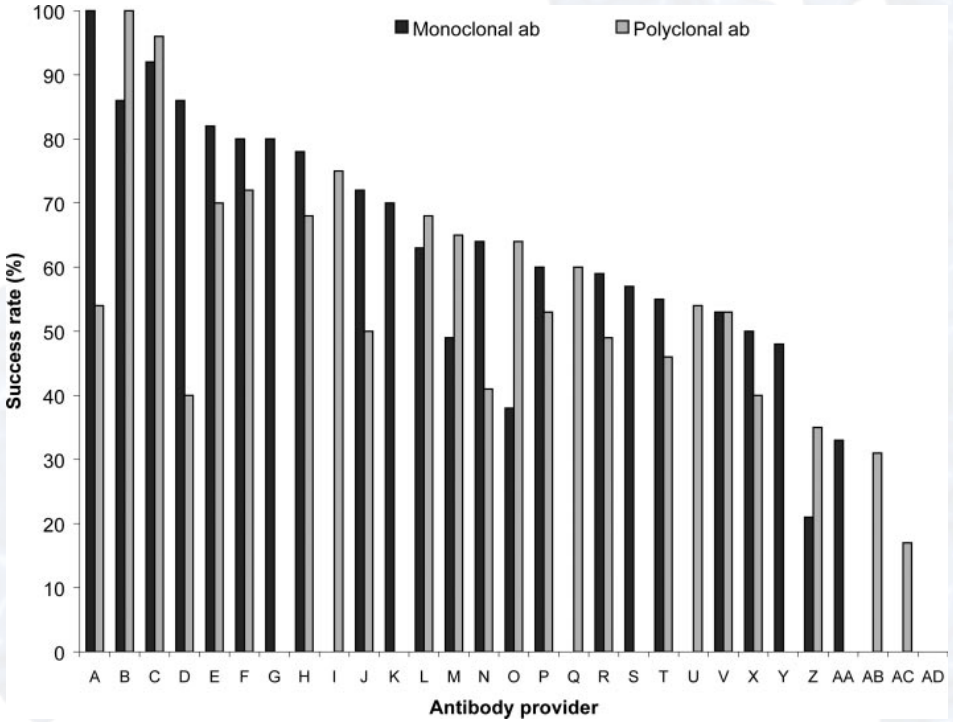
whatever the manufacturer claims is not consistent with good science.

ANTIBODY SOCIETY

Can the extent of the problem be quantified?

Molecular & Cellular Proteomics 7.10 2019

A Genecentric Human Protein Atlas for Expression Profiles Based on Antibodies



as described above. The success rates stratified by the different providers (Fig. 2) showed large differences, ranging from 0 to 100% of the antibodies with an average success rate of 49%. It is important to point out that many of these antibodies have not been approved by the antibody providers for immunohisto-chemistry; this might explain the low success rate in our hands.

NATURE|Vol 447|7 June 2007 TECHNOLOGY FEATURE **ANTIBODIES**

The generation game

Proteomics is hungry for well-validated antibodies. Nathan Blow looks at the options and sees how researchers are redefining the way to generate an antibody.

In addition to producing and testing their own antibodies, **human proteo**
Uhlén and his team will put antibodies from commercial sources through their standardized quality-control pipeline. Uhlén says he was surprised that only about 35% of commercial antibodies seemed to work — although he notes that this could be a result of the way his group analyses them. "We decided to use a very standardized way of validating antibodies: if they don't work, we don't try other ways of doing it," he says. The success rate may be low,

Protein microarrays and proteomics

Gavin MacBeath **nature genetics supplement • volume 32 • december 2002**

In experiments carried out in collaboration with Peter Sorger, my research group has found that it is much more difficult to find antibodies to study intracellular proteins. In fact, only about 5% of over 100 commercial antibodies that we have tested are suitable for microarray-based analyses of cellular lysates (U.B.

Depending upon the specific assay, only 5-49% of commercial antibodies work

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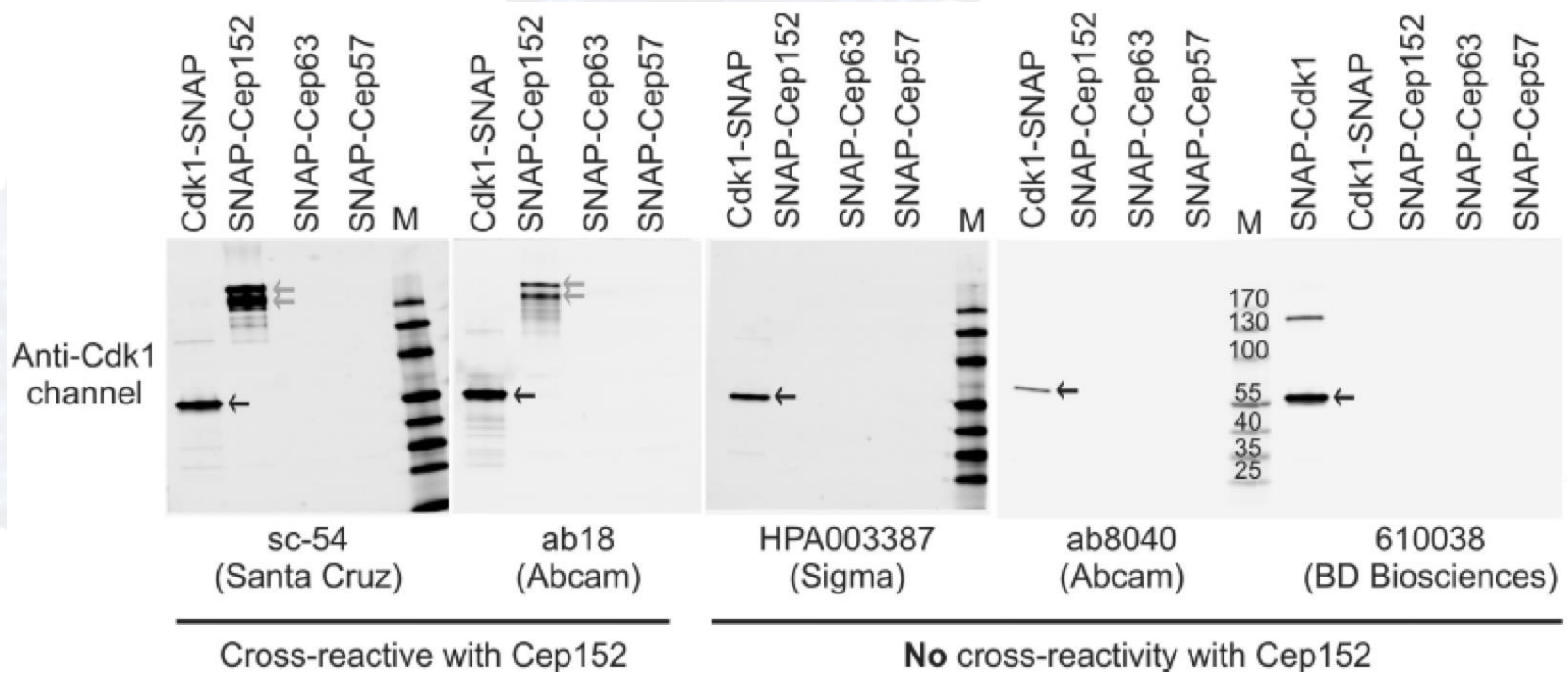
A recent *published* example Used in over 200 papers

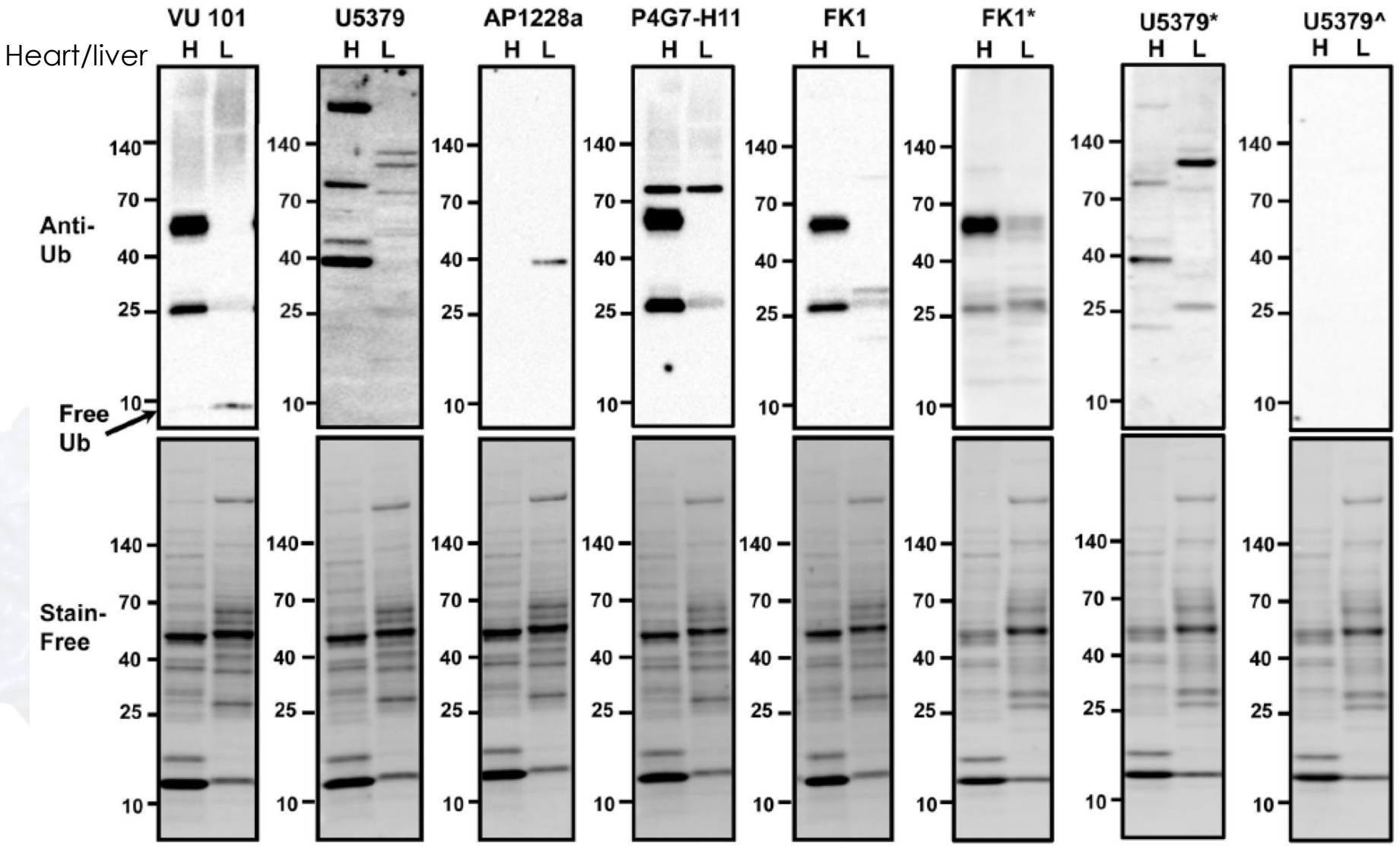
- Sold as recognizing Cdk1, also recognizes Cep152



Lukinavicius et al., (2013). Biotechniques **55**(3): 111-114.

ANTIBODY SOCIETY

Antibodies against ubiquitin



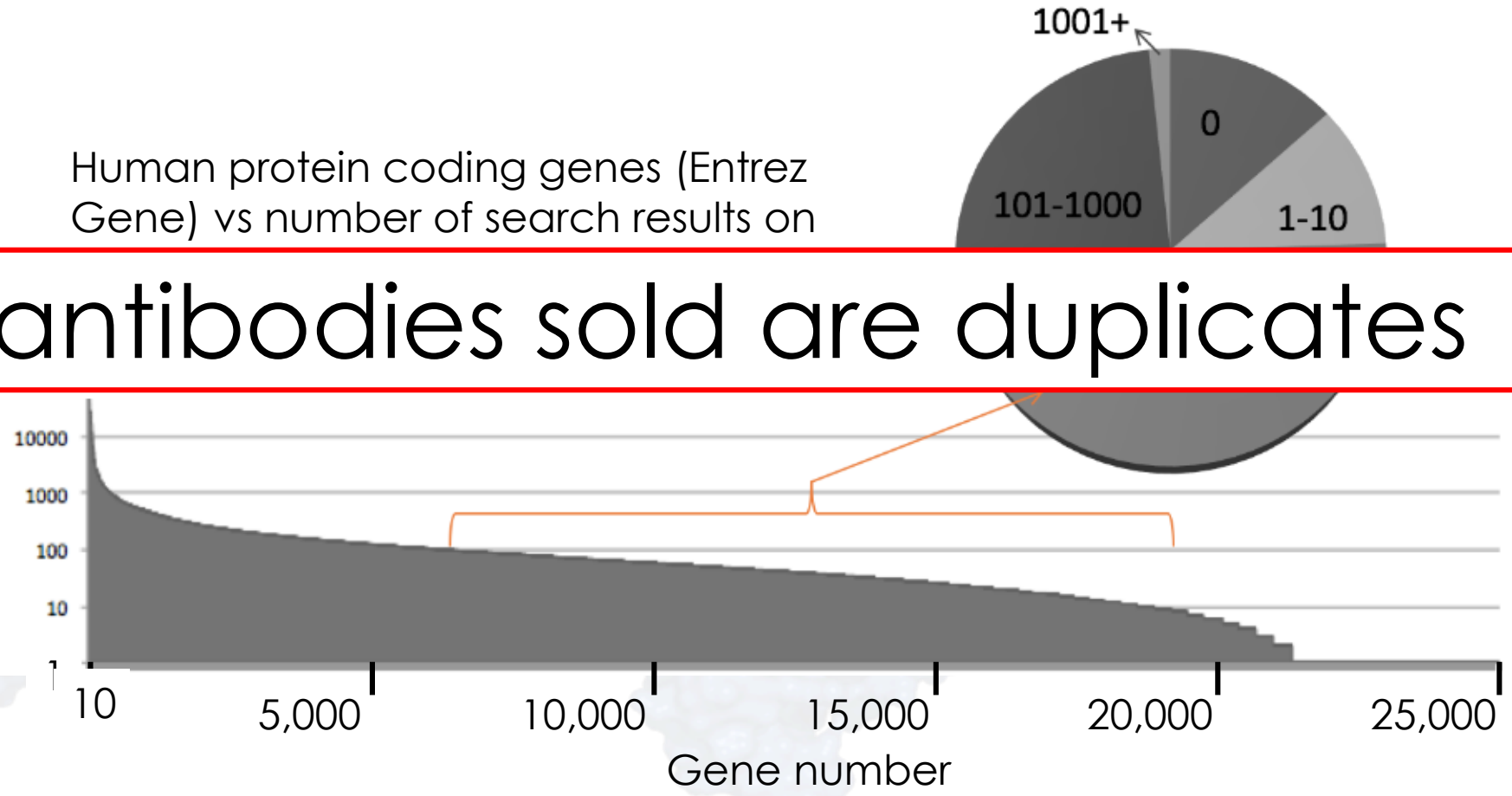
Gilda, J. E. et al. PLoS One **10**, e0135392, (2015).

ANTIBODY SOCIETY

How many antibodies actually out there?

Human protein coding genes (Entrez Gene) vs number of search results on

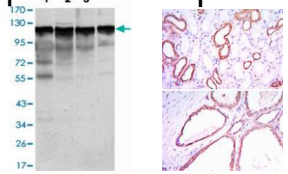
M
re
phosphatase C



Most antibodies sold are duplicates

382 monoclonal antibodies against hexokinase 1 from 27 providers on Antibodypedia

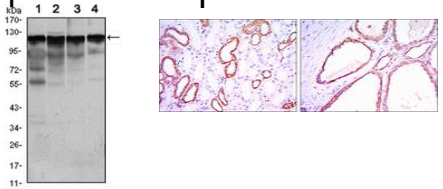
Abnova – MAB10683
$335 100µl



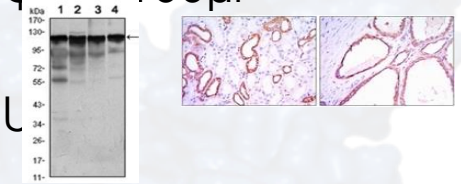
Acris Antibodies AM06498SU
$330 100µl

Novus Biologicals NBP1-5164
$349 100µl

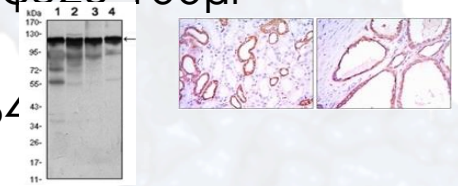
GeneTex GTX82790
$299 100µl



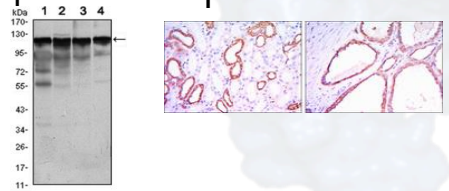
ThermoFisher MA5-15680
$324 100µl



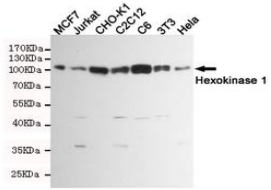
Abgent AO1436a
$325 100µl



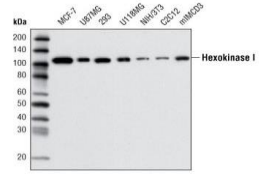
Biorbyt orb69303
$330 100µl



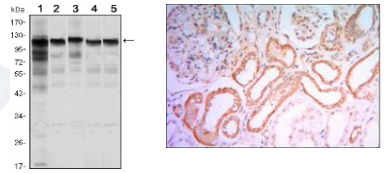
Arigo ARG54058
$299 100µl



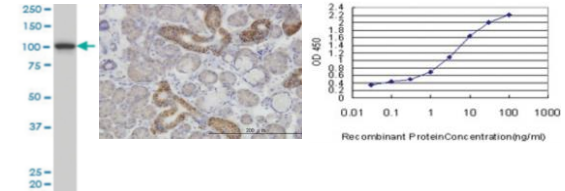
CST 2024S
$239 100µl



LSBio LS-C169235
$345 100µl



Sigma 5G9 (discontinued)
$347 100µg



ANTIBODY SOCIETY

What's the problem?

- Polyclonals are practically undefinable
- Monoclonals are unknown molecular entities
  - ~35% of monoclonals have additional expressed chains: may contribute to off-target binding*
  - Recombinant antibodies derived from hybridomas almost always have improved activity and reduced off-target binding over original mAb*
  - Monoclonals cell lines:
    - can continue to mutate
    - may die out, or change following necessary recloning
    - institutions (e.g. Scripps) may discard a researcher's life work, including their hybridomas, once they retire
- Lot-to-lot variation: impossible to know if two batches are the same
- Data sheets are historical: do not usually correspond to supplied lots
- Antibodies are sold on the basis of what they (purportedly) recognize, not their physical identity (what they are)

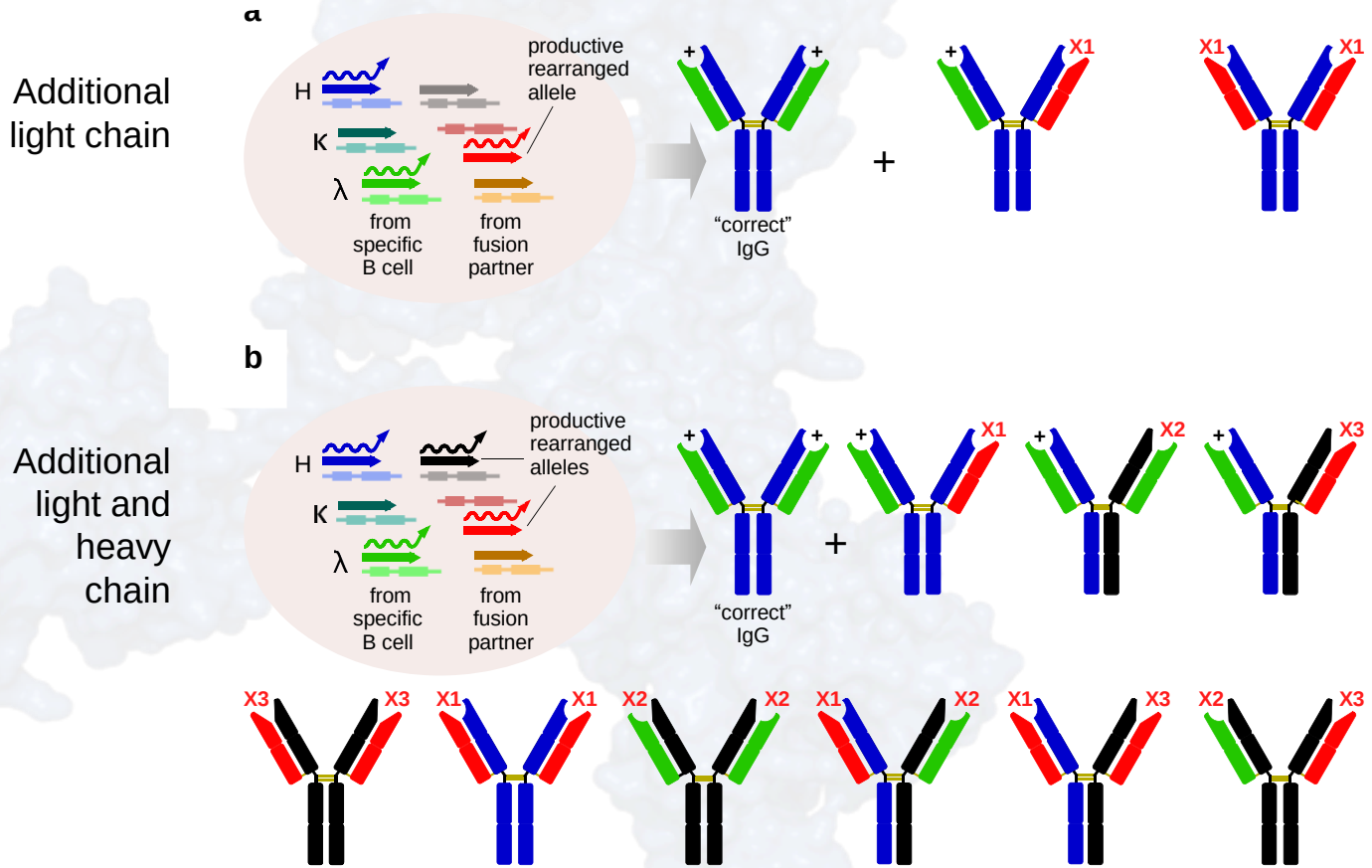
Bradbury et al., (2018). MAbs **10**(4): 1-19; Bradbury and Pluckthun (2015) Nature **518**(7537): 27-29;
Bradbury, A. R. and A. Pluckthun (2015). Protein Eng Des Sel **28**(10): 303-305.

ANTIBODY SOCIETY

How do you choose a good antibody?

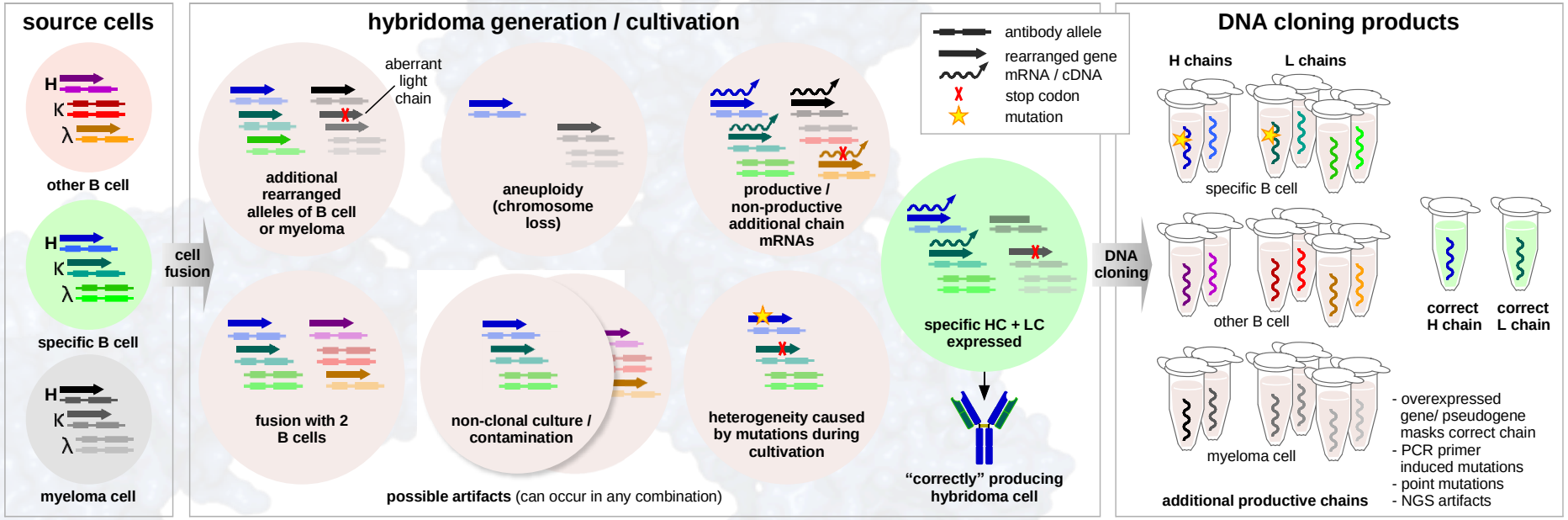
- Impossible to test all antibodies, and many are duplicates, anyway (and difficult to work that out)
- Citations
- Human Protein Atlas
  - >22,000 antibodies against >14,000 different targets
  - Primary data presented – make your own mind up
    - Limited numbers of antibodies are tested
  - Immunohistochemistry, immunofluorescence, western blot, protein arrays, normal and tumor tissues, cell lines

The effect of multiple chains



Bradbury et al., (2018). MAbs **10**(4): 1-19.

How extra chains arise



Bradbury et al., (2018). MAbs **10**(4): 1-19.

Percentage of hybridomas containing additional chains: NGS+ traditional cloning

Class	Numbers	Percentage
Correct VH/VL, no additional chains	126/185	68.1
Additional productive VL	53/185	28.6
Additional productive VH	2/185	1.1
Additional productive VL+VH	4/185	2.2

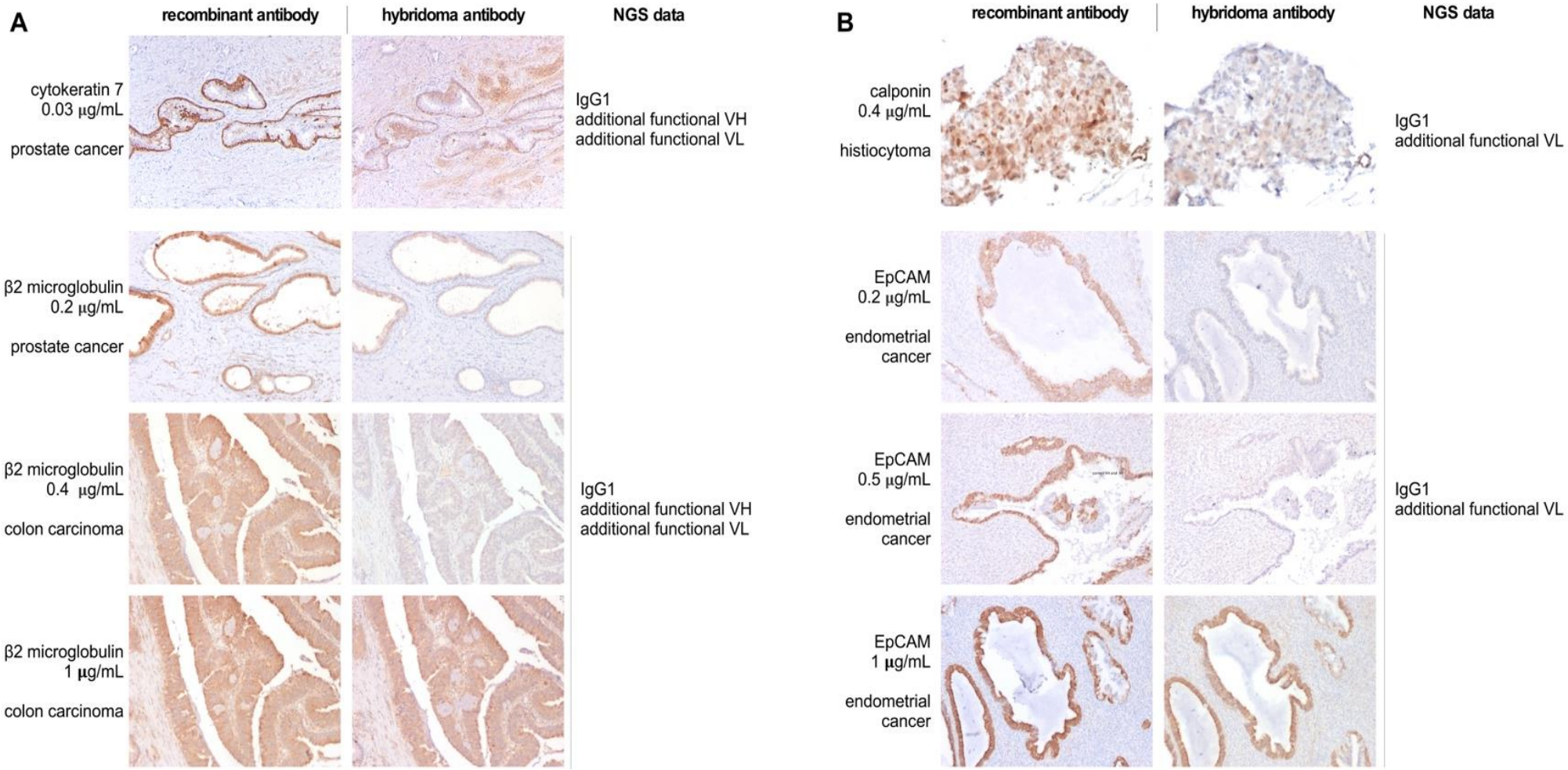
Bradbury et al., (2018). MAbs **10**(4): 1-19.

Recombinant antibodies give much stronger signals than the original hybridomas

Target name	Active Motif prod no	Purified IgG	EST CONC (ng/mL)	EED	EZH2	Hh3	Hh3.3	P300	POL2 p0	POL2 pS2	POL2 pS5
EED	61203.0	Hybridoma1	1000.0	146.6	258.8	0.7	0.7	0.8	0.6	0.8	0.8
		Hybridoma1	100.0	9.0	31.3	0.6	0.6	0.7	0.5	0.7	0.6
		VH1 VL1	20.0	104.9	126.0	1.4	1.4	0.9	0.9	0.7	0.8
EZH2	39875.0	Hybridoma2	1000.0	0.2	301.6	4.1	1.2	1.0	0.5	0.5	0.5
		Hybridoma2	100.0	0.4	35.4	0.7	0.7	0.9	0.6	0.7	0.7
		VH1 VL1	20.0	0.3	432.4	9.3	4.2	0.5	0.5	0.5	0.5
		VH2 VL1	20.0	0.5	1.2	1.6	2.1	0.5	0.5	0.5	0.5
SUZ12	39877.0	Hybridoma3	1000.0	0.7	337.9	1.8	6.0	1.8	1.3	1.5	1.7
		Hybridoma3	100.0	0.6	32.7	1.0	1.0	1.6	1.0	1.2	1.3
		VH1 VL1	20.0	0.4	82.2	1.2	1.3	0.7	0.6	0.6	0.6
		VH2 VL2	20.0	0.3	8.6	1.6	2.0	73.4	3.9	105.2	274.5
		VH1 VL2	20.0	0.4	1.7	1.3	1.5	0.8	0.6	1.5	2.1
		VH1 VL3	20.0	0.4	0.9	1.5	1.3	0.7	0.7	0.6	0.7
		VH2 VL3	20.0	0.3	0.7	0.9	1.0	0.5	0.5	0.6	0.8
		VH2 VL1	20.0	0.3	0.9	3.1	0.9	0.5	0.7	0.5	1.1
H3total	61475.0	Hybridoma4	900.0	0.6	18.6	60.0	53.5	1.4	1.3	1.3	1.4
		Hybridoma4	90.0	0.6	16.9	2.0	1.8	1.8	1.2	1.3	1.4
		VH1 VL1	20.0	0.6	2.0	58.8	42.2	1.2	1.4	1.3	1.3
		VH1 VL2	20.0	0.5	1.3	2.1	1.7	0.9	1.1	1.0	1.0
pol2	39097.0	Hybridoma6	200.0	2.3	25.1	0.9	1.0	3.9	1.1	15.8	44.9
		Hybridoma6	20.0	0.5	18.5	0.8	0.8	1.1	0.8	1.1	1.4
		VH1 VL1	20.0	0.6	8.5	2.3	3.3	55.1	9.8	71.9	274.8
		VH2 VL1	20.0	0.3	0.9	1.3	1.6	1.4	0.6	0.7	3.2
pol2ser2	61083.0	Hybridoma7	1000.0	0.4	17.9	0.7	0.7	1.0	0.6	29.6	0.8
		Hybridoma7	100.0	0.4	16.1	0.6	0.7	1.3	0.7	0.9	0.8
		VH1 VL1	20.0	0.2	1.3	1.1	1.2	0.7	0.7	424.9	1.5
		VH1 VL2	20.0	0.3	0.7	0.9	1.1	0.6	0.4	0.5	0.6
		VH1 VL3	20.0	0.2	1.3	1.0	1.2	0.4	0.4	0.5	0.5
pol2ser5	61085.0	Hybridoma8	1000.0	0.4	15.8	0.8	0.9	1.0	1.4	21.8	11.7
		Hybridoma8	100.0	0.3	17.2	0.8	0.8	1.2	0.8	1.2	1.0
		VH1 VL1	20.0	0.2	0.7	1.3	1.2	1.2	1.9	288.1	261.3
		VH1 VL2	20.0	0.3	1.2	1.5	1.5	0.8	0.6	1.8	447.5

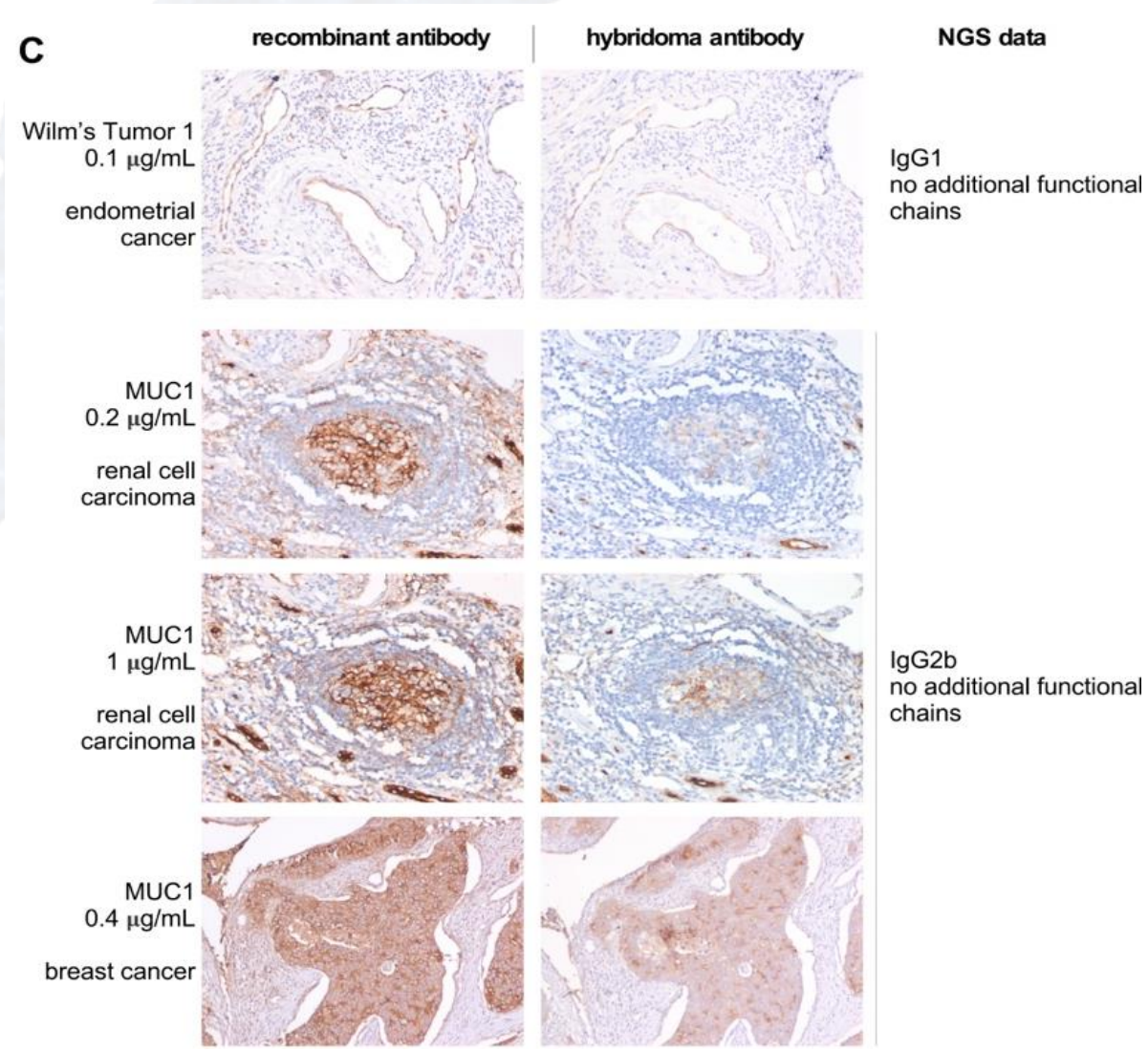
Correct Ab/Ag combination

Recombinants provide improved activity



Bradbury et al., (2018). MAbs **10**(4): 1-19.

…even if no additional chains are identified



Bradbury et al., (2018). MAbs **10**(4): 1-19.

ANTIBODY SOCIETY

Validate that the correct VH and VL have been cloned

- Fastest to express as scFv or Fab fragment
  - Secreted from bacteria: purify and measure K_D
  - Displayed on yeast: K_D measured
- Alternatively transiently express as IgG: measure K_D
- If K_D different than wild type, likely have not isolated correct V-genes
- If multiple chains, need to clone and test all variations

Natalie de Souza (editor Nat. Methods)

"…Antibody companies sell products they say are the same, that are different, and products they say are different, that the same…"

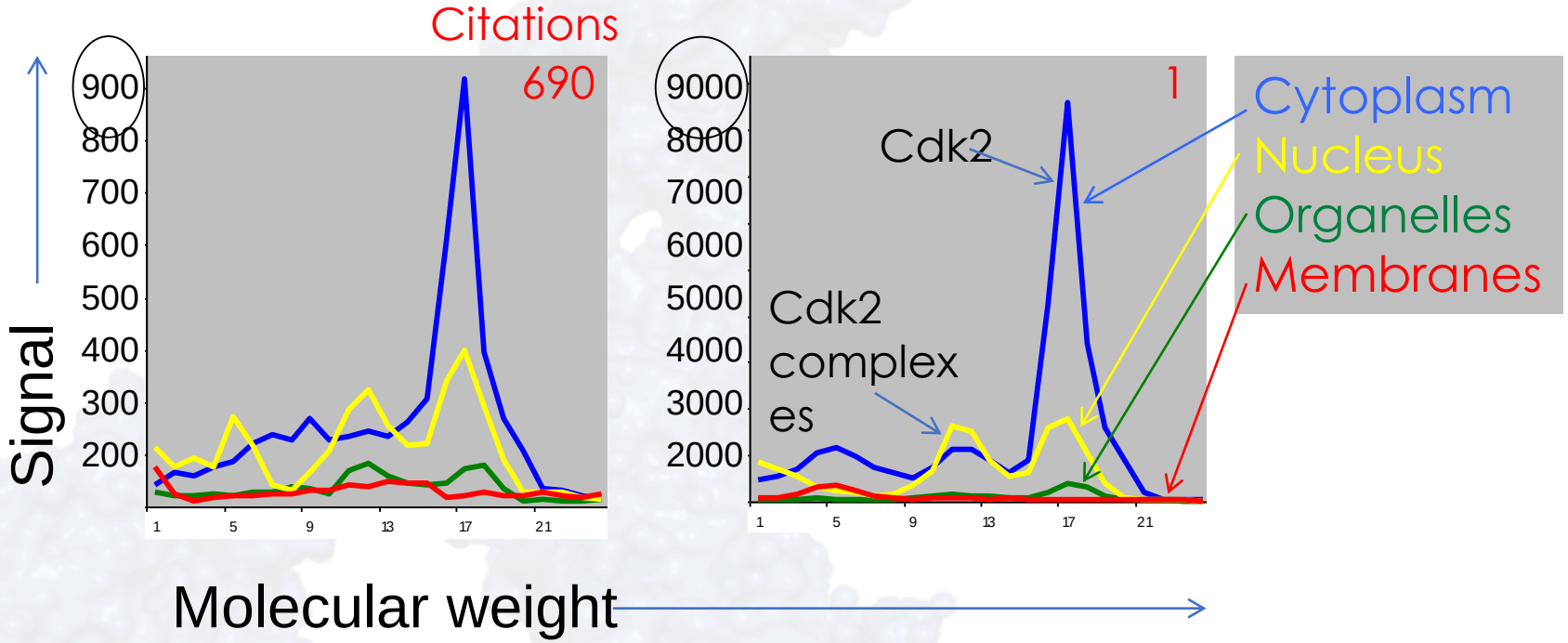
ANTIBODY SOCIETY

Can you rely on citations to pick the best? Santa Cruz Biotechnology Cdk2 antibodies

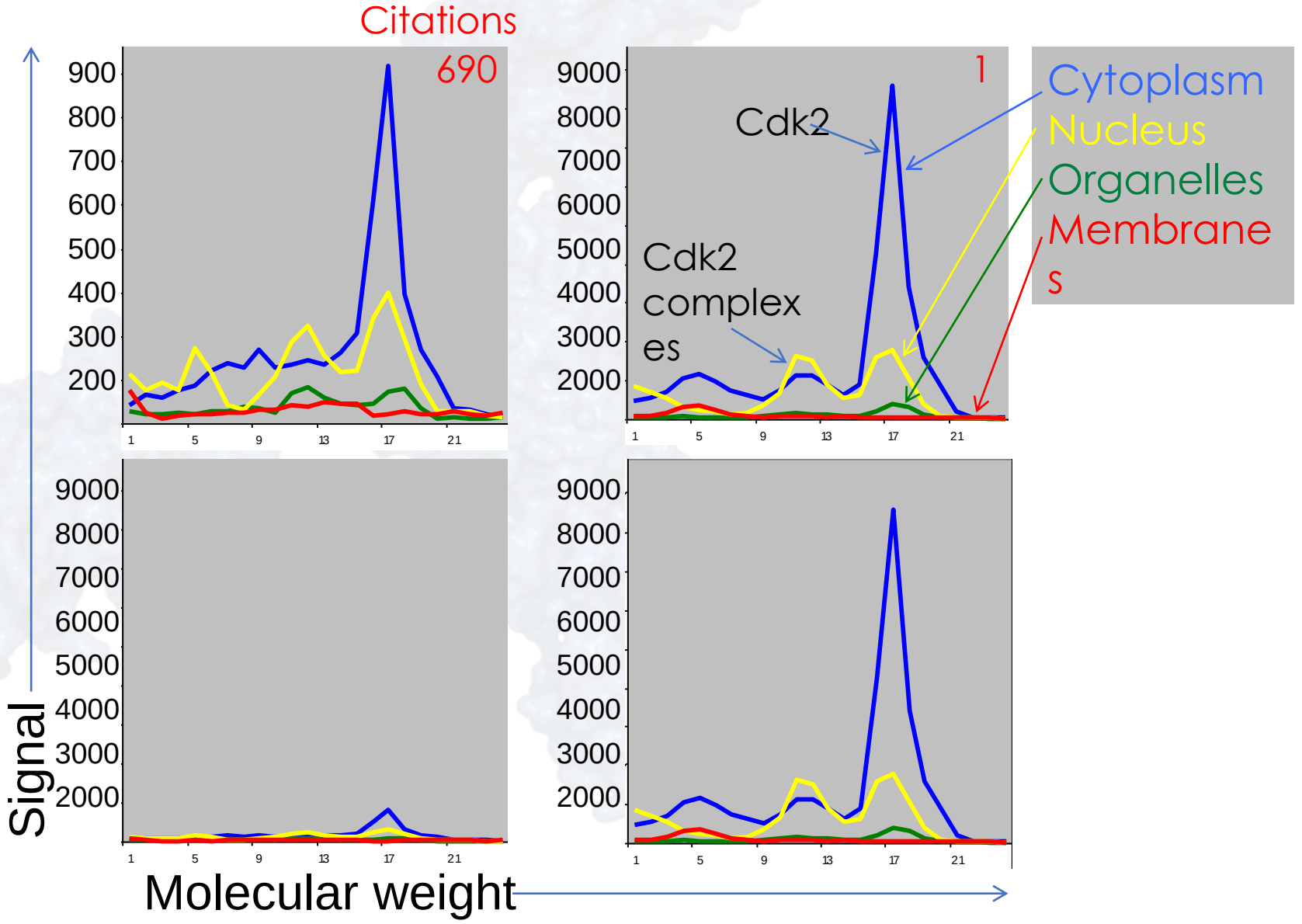
PRODUCT NAME	CAT. #	ISOTYPE	EPITOPE	APPLICATIONS	SPECIES	CITATIONS
Cdk1/Cdk2 (AN21.2)	sc-53219	mouse IgG_{2a}	FL (h)	WB, IP, IF, IHC(P)	m, h	6
Cdk2 (D-12)	sc-6248	mouse IgG_1	FL (h)	WB, IP, IF, ELISA	m, r, h	125
Cdk2 (H-298)	sc-748	rabbit IgG	FL (h)	WB, IP, IF, ELISA	m, r, h, e, c, b, p, a	58
Cdk2 (M2)	sc-163	rabbit IgG	C-terminal (h)	WB, IP, IF, KA, ELISA	m, r, h, hamster, e, c, b, p	690
Cdk2 (AN4.3)	sc-53220	mouse IgG_{2a}	FL (h)	WB, IP	m, h	5
Cdk2 (0.N.198)	sc-70829	mouse IgG_{2a}	FL (h)	WB, IP	m, h	2
Cdk2 (55)	sc-136191	mouse IgG_{2a}	N/A	WB, IP, IF	m, r, h	1

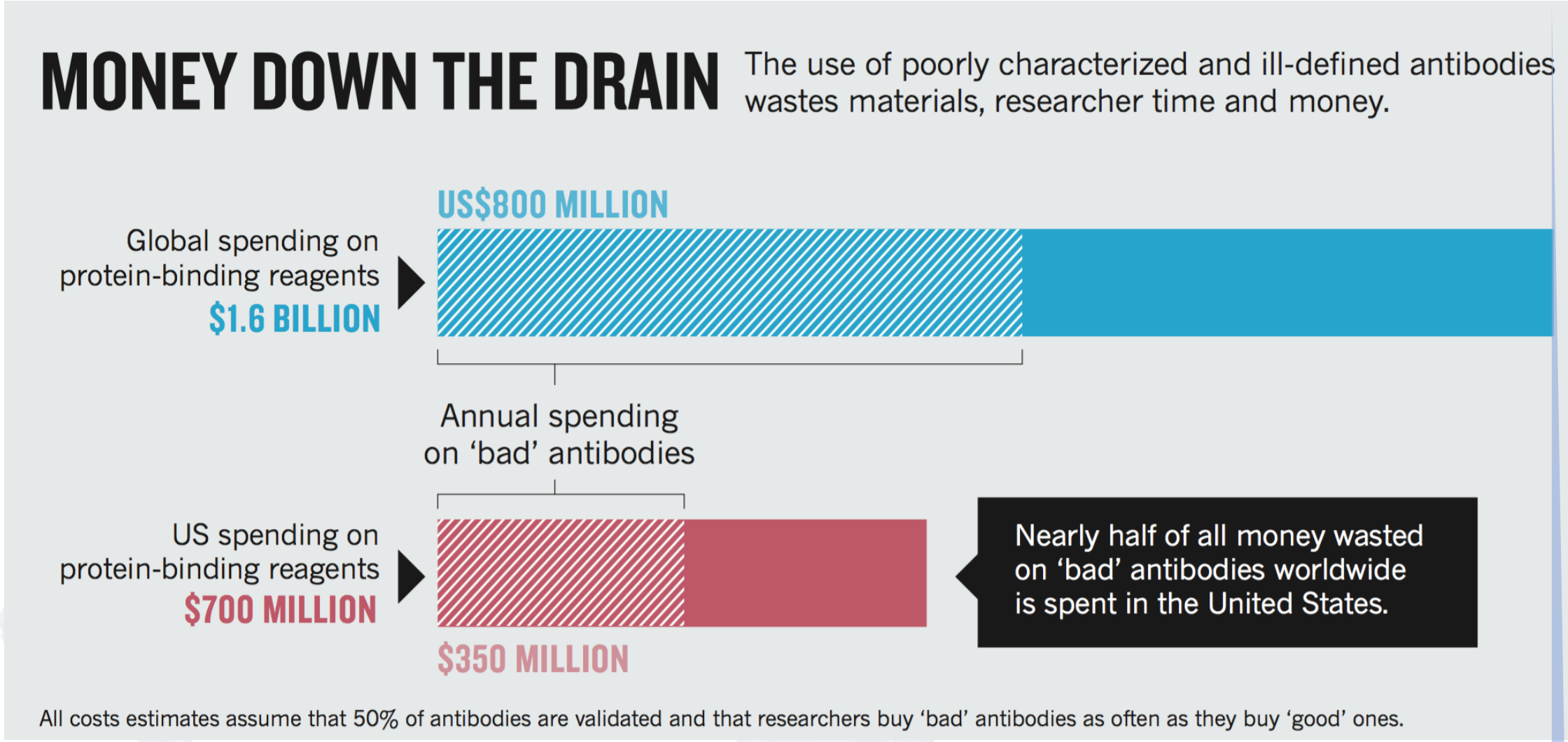
ANTIBODY SOCIETY

Can you rely on citations to pick the best? #2



The tyranny of the first antibody to publish or first mover advantage





Takes no account of other reagent costs, wasted time, follow-on research or possible even lower estimates of functionality

Bradbury, A. & Pluckthun, A. Reproducibility: Standardize antibodies used in research, *Nature* **518**, 27, (2015).

ANTIBODY SOCIETY

How to solve this problem?

Average customer:

"We need better antibodies"

Average manufacturer:

"People will not buy anything but traditional mAbs"

Henry Ford:

"If I had asked people what they wanted, they would have said ‘faster horses’."

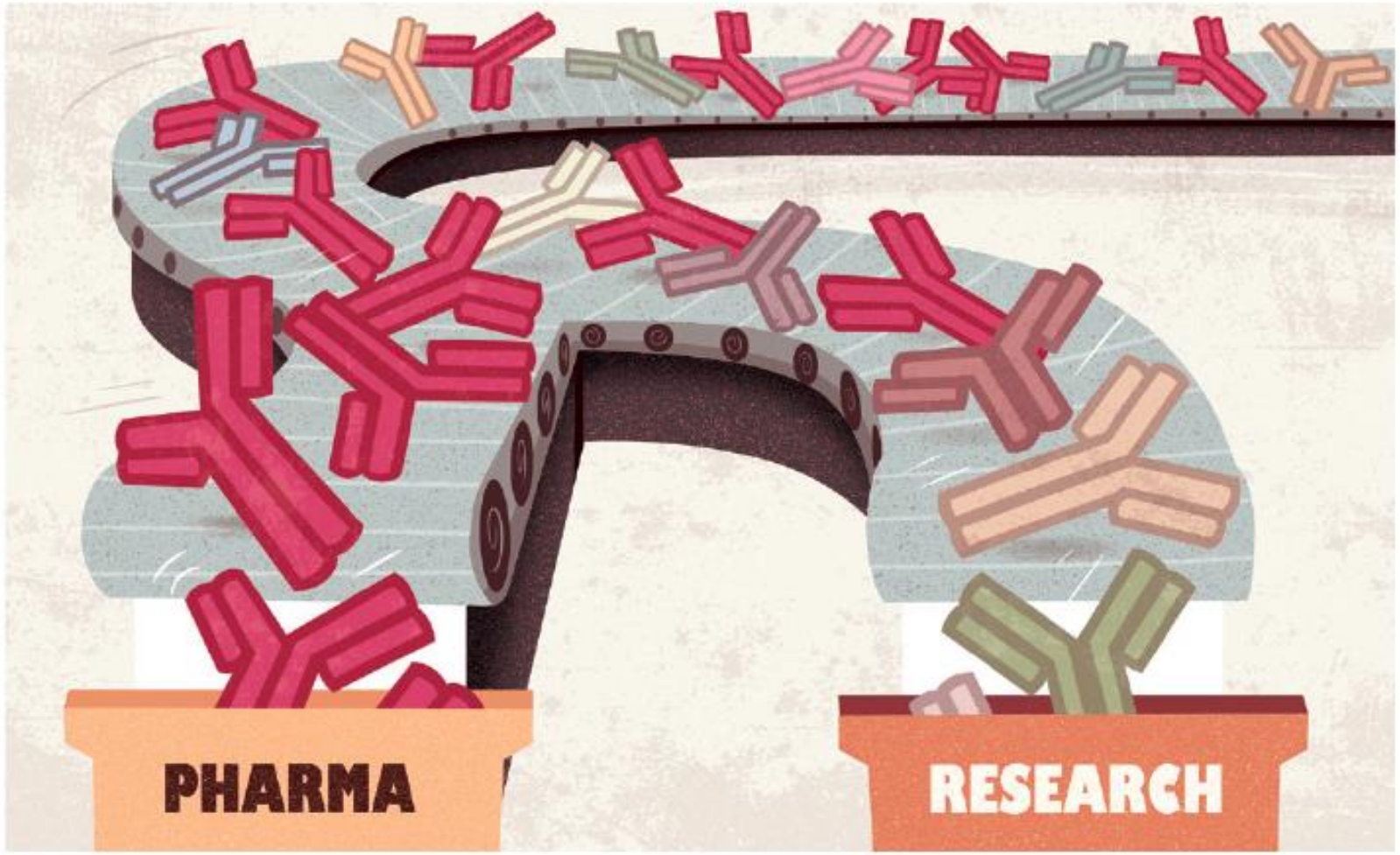
Steve Jobs:

"It's not the consumer's job to know what they want"

"People don't know what they want until you show it to them"

What's the conventional solution?

- "Antibodies need be better characterized and then all is well"
- But: even well-characterized good antibodies are not defined or archived forever
  - Polyclonals: practically undefinable
  - Monoclonals: unknown molecular entities
    - ~35% with additional expressed chains
    - They may mutate
    - Recombinant antibodies ex-mAb : better activity. Less off-target binding
  - Cell lines may die, or need recloning
  - Institutions discard departed researcher's hybridomas
  - Lot-to-lot variation – are any two batches the same?
  - Data sheets historical: not usually describing to the lots supplied



Standardize antibodies used in research

To save millions of dollars and dramatically improve reproducibility, protein-binding reagents must be defined by their sequences and produced as recombinant proteins, say **Andrew Bradbury**, **Andreas Plückthun** and 110 co-signatories.

ANTIBODY SOCIETY

Antibodies: validate recombinants once

It goes without saying that recombinant antibodies, like all binding reagents, need to be validated at the outset (see R. D. Polakiewicz *Nature* **518**, 483 (2015) and L. P. Freedman *Nature* **518**, 483 (2015)). However, we anticipate that recombinant antibodies will require only one such extensive characterization — unlike conventionally raised antibodies.

This single validation will assure scientists that antibodies with identical sequences will have similar reactivity profiles — subject to routine checks that binding activity has not been compromised during transit or by storage conditions.

We are aware that our proposal is incompatible with current business models for commercial reagent antibodies. We do not believe that the answer is to defend the status quo, which has not served science well (A. Bradbury and A. Plückthun *Nature* **518**, 27–29; 2015). The solution is to develop more imaginative business strategies that are compatible with the marketing of fully validated, publicly available recombinant antibody sequences.

Andrew M. Bradbury *Los Alamos National Laboratory, New Mexico, USA.*
Andreas Plückthun *University of Zurich, Switzerland.*
amb@lanl.gov

- Antibodies should be well characterized and validated
- Antibodies should be expressed recombinantly and identified by unique "bar codes": their publicly available sequences
- Characterization will be required only once if everyone is using the same recombinant sequenced reagents
  - Functionality needs to be tested on each lot

ANTIBODY SOCIETY

Antibodies: the solution is validation

I disagree with Andrew Bradbury and colleagues' suggestion that making the sequences of commercial antibodies publicly available could minimize irreproducibility in biomedical research (*Nature* **518**, 27–29; 2015). The real solution is proper initial validation of antibodies.

In my view, the reproducibility problem is better addressed by identifying the good antibodies and the reputable companies that develop, validate and manufacture them — as astute scientists do now. Also, journals need to mandate the provision of detailed validation data, protocols and antibody sources (clone, catalogue number). Independent websites enabling the submission of antibody data and consumer feedback would also help.

The biggest investment in developing a good monoclonal antibody is the extensive work needed to validate specificity and sensitivity across all relevant applications. Unlike therapeutic antibodies, most research antibodies are not sequence-patented because the cost is too high to be recovered by sales.

Even if the practical hurdles of funding and enforcing a sequence-publishing policy could be overcome, making unpatented antibody sequences public would allow them to be widely copied, produced and sold. This would eliminate the incentive for good companies to invest in validation. It would also allow 'bad' antibody sequences to contaminate the databases.

The authors' proposal could therefore disproportionately harm the good companies, hurt the end-users it is designed to protect, and would not solve the reproducibility problem.

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The company response

- Antibodies should be properly validated
- Identify good antibodies and reputable companies
- Journals should mandate disclosure of detailed validation data and antibody sources (clone, catalog and lot number)
- Public sequences would enable easy copying

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Company concerns

- Antibody sequences represent IP
  - If published, others will use them
  - Research antibodies don't make enough money to patent, so they will be unprotected
  - There are no cheap ways to protect antibody sequences
- Research antibodies don't make much money, not worth investing in additional technology to make recombinants

How to address these concerns

- Fund (public or public/private) specialized centers for binder selection, sequencing and characterization
  - Companies compete on the production of publicly available sequence validated antibodies
- Technology to generate, characterize and produce antibodies becomes so cheap that publication of sequence is no longer a concern
  - E.g. oligonucleotides
- Third party escrow of antibody sequences (not very satisfactory)
  - All producers of recombinant antibodies refer to each antibody using a code
  - Users (scientists) do not know the sequences, but know antibodies with the same code have the same sequence

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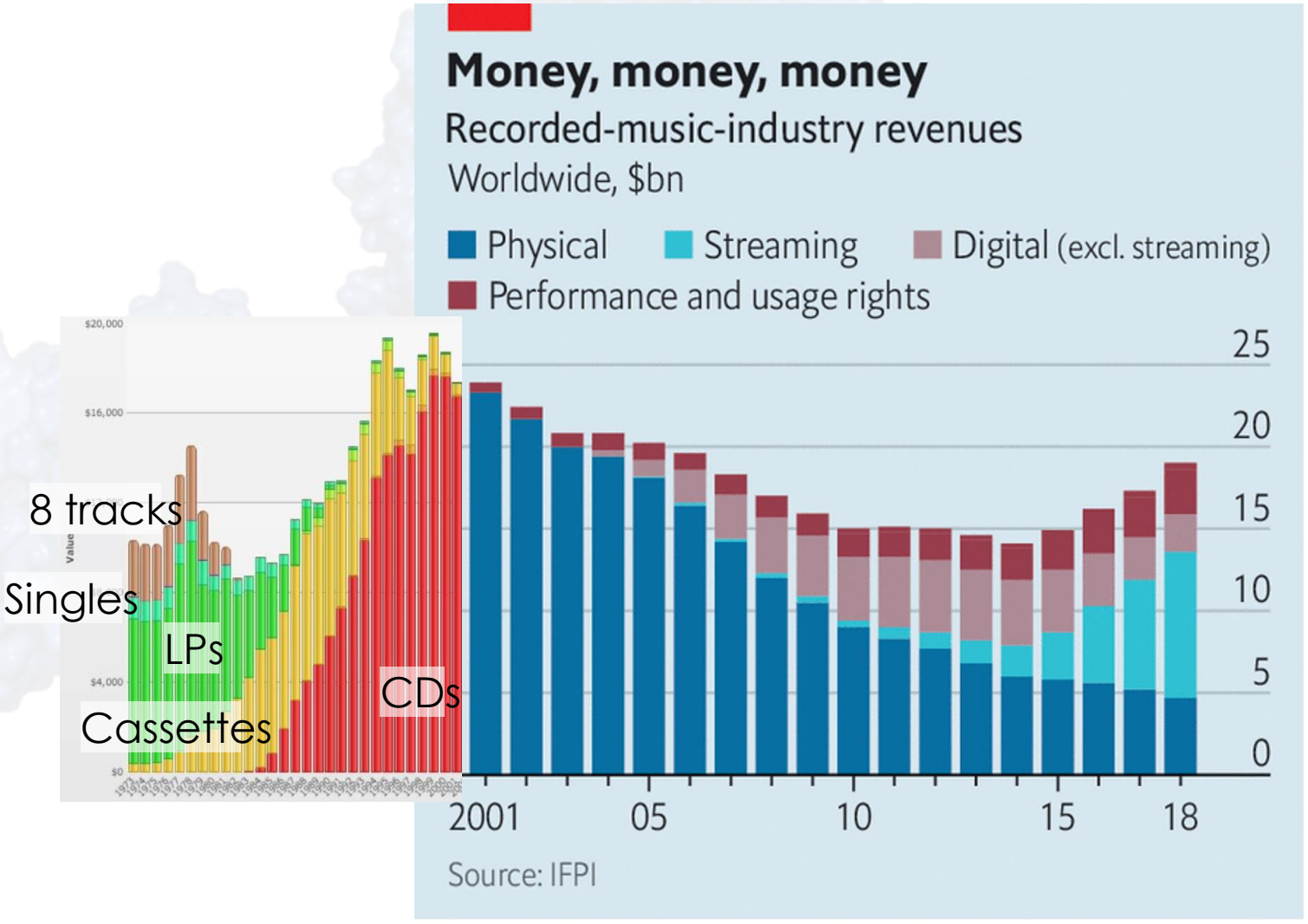
The power of sequence based antibody definition

- Genes, mRNA, proteins, oligos, siRNA defined by sequence
  - Why should antibodies be a special case?
- I can repeat/reproduce your experiments using the same reagents
- Good binders will become immortal
  - The genes and hence antibodies can always be resynthesized
- As binder citations accumulate, complete understanding of the properties of that binder
- Complete characterization is required only once
  - After that important to confirm binding activity (e.g. not lost on poor storage) but similar properties can be assumed

Additional advantages of sequenced defined binding reagents (not just antibodies)

- Eventual possibility of web-based distribution of antibody sequences and gene and antibody synthesis in house
- Different Fc domains (IgA, IgG, IgM, IgE) from different species
- Incorporation of binding reagents into multifunctional domains
  - Enzymes (e.g. alkaline phosphatase) allowing one step detection
  - Fluorescence
  - Bi- or multispecific binding agents
  - Intracellular knockouts
  - Viral retargeting

The music industry as a victim of disruption

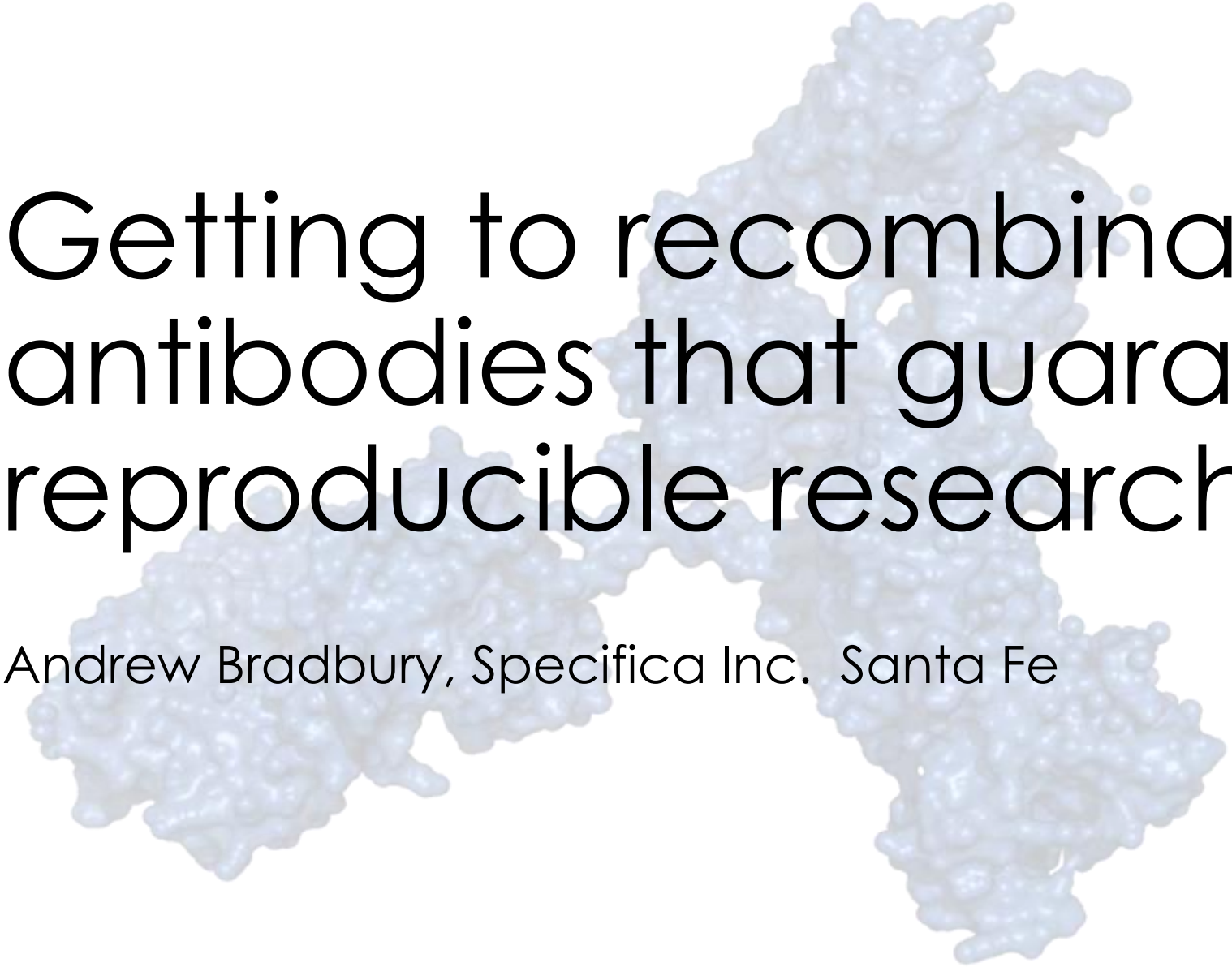


Conclusion

- Apply the gene-based paradigm to antibodies
- Time for a new business model
- In vitro methods generate good sequenced recombinant antibodies
- Using an unsequenced antibody should become as unacceptable as using an unsequenced plasmid, oligo, gene etc.
- Sequence defined binding reagents have many more advantages

Getting to recombinant antibodies that guarantee reproducible research

Andrew Bradbury, Specifica Inc. Santa Fe



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