ROCKLAND™
antibodies & assays

ANTIBODY BASICS

FOR THE PRODUCTION OF FUNCTIONAL ANTIBODIES

Spring, 2016

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Antibody Basics

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#1 - Pick the Right Immunogen

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- Native protein vs. recombinant protein vs. synthetic peptide?
  - Each has pros and cons that will affect antibody binding.
- If planning on immunizing a peptide, have you considered:
  - Potential cross reactivity between human and mouse?
  - Internal vs surface epitope?
  - Isoform homology? Desired? Undesired?
  - Critical post translational modifications
- Where are potential sites of glycosylation?
- What about targeting a domain of interest?
- Is the sequence immunogenic? Soluble?
- Blockers for internal sequences?
- Have you considered linkers (AHX) to facilitate coupling and better presentation?

"Thoughtful design."

#1 - Pick the Right Immunogen

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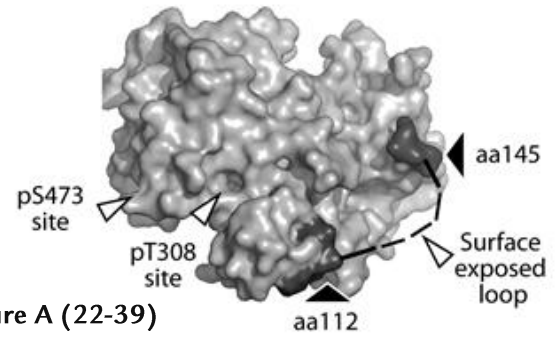
```
                5         10        15        20        25        30
                                                    capture A
 1 M S D V A I V K E G W L H K R G E Y I K T W R P R Y F L L K
                                                    capture B
31 N D G T F I G Y K E R P Q D V D Q R E A P L N N F S V A Q C
                                                    capture B
61 Q L M K T E R P R P N T F I I R C L Q W T T V I E R T F H V
                                                    capture C
91 E T P E E R E E W T T A I Q T V A D G L K K Q E E E E M D F
                                                    capture C
121 R S G S P S D N S G A E E M E V S L A K P K H R V T M N E F
151 E Y L K L L G K G T F G K V I L V K E K A T G R Y Y A M K I
181 L K K E V I V A K D E V A H T L T E N R V L Q N S R H P F L
211 T A L K Y S F Q T H D R L C F V M E Y A N G G E L F F H L S
241 R E R V F S E D R A R F Y G A E I V S A L D Y L H S E K N V
271 V Y R D L K L E N L M L D K D G H I K I T D F G L C K E G I
                pT308 epitope
301 K D G A T M K T F C G T P E Y L A P E V L E D N D Y G R A V
                  (p)
                                                    capture D
331 D W W G L G V V M Y E M M C G R L P F Y N Q D H E K L F E L
361 I L M E E I R F P R T L G P E A K S L L S G L L K K D P K Q
391 R L G G G S E D A K E I M Q H R F F A G I V W Q H V Y E K K
421 L S P P F K P Q V T S E T D T R Y F D E E F T A Q M I T I T
                                                    pS473 epitope
451 P P D Q D D S M E C V D S E R R P H F P Q F S Y S A S G T A
                                      (p)           S
```

AKT1 surface representation



Capture A (22-39)

hAKT1	WRPRYFLLKNDGTFIGYK
hAKT2	WRPRYFLLKSDGSFIGYK
hAKT3	WRPRYFLLKTDGSFIGYK

Capture B (77-90)

hAKT1	LQWTTVIERTFHV
hAKT2	LQWTTVIERTFHV
hAKT3	LQWTTVIERTFHV

Capture C (134-146)

hAKT1	MEVSLAKPKHRVT
hAKT2	MEVAVSKARAKVT
hAKT3	MDASTTHHK-RKT

Capture D (346-361)

hAKT1	CGRLPFYNQDHEKLFE
hAKT2	CGRLPFYNQDHERLFE
hAKT3	CGRLPFYNQDHEKLFE

#1 - Pick the Right Immunogen

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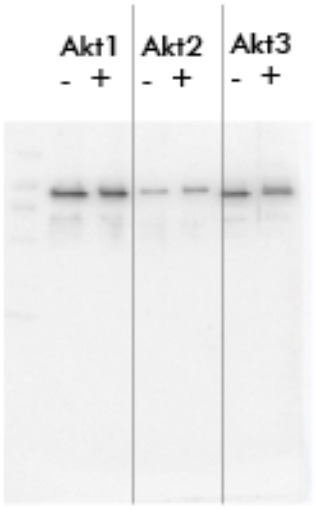
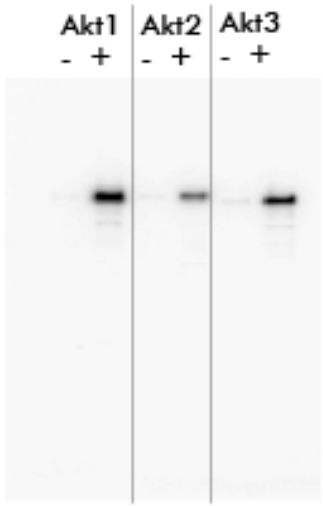
Rb α-AKT (pan)



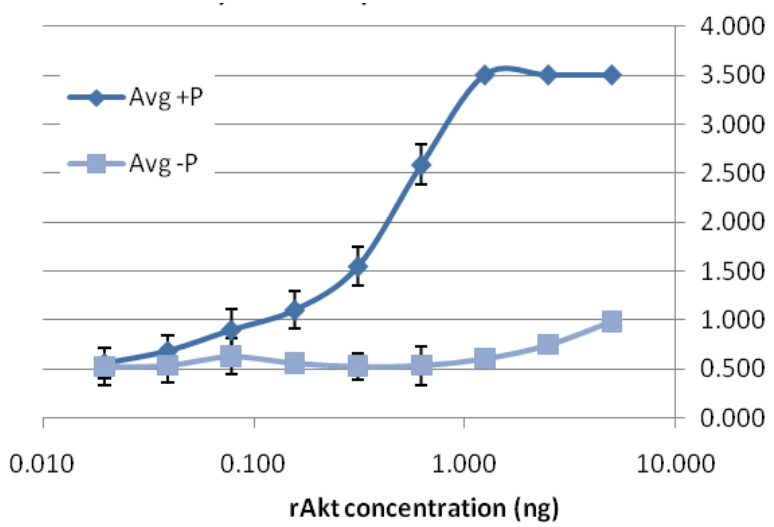
Anti- Capture B (77-90)

Ms α-AKT pT308



Anti- pT308

Ms α-AKT pS473



cAb = Capture B (77-90)
dAb = Anti- pS3473

#2 - Know your Intended Assay

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Begin with the end. Assays have vastly different Ab requirements.

- Consider that an antibody screened for by western blotting will likely be of little value if you intend to use the antibody for a native assay like immunoprecipitation, capture ELISA or frozen section immunohistochemistry.
- Platform based assays have demanding requirements for antibody performance (i.e. extremely low background and cross-reactivity) like:
  - multi-array, multi-specific electrochemiluminescence assays like MSD,
  - charge and size based separations like Protein Simple's Peggy Sue, and
  - multiplex systems with laser and LED emitters and photo detectors like Luminex.

MSD® Meso Scale Discovery

Luminex®

#2 - Know your Intended Assay

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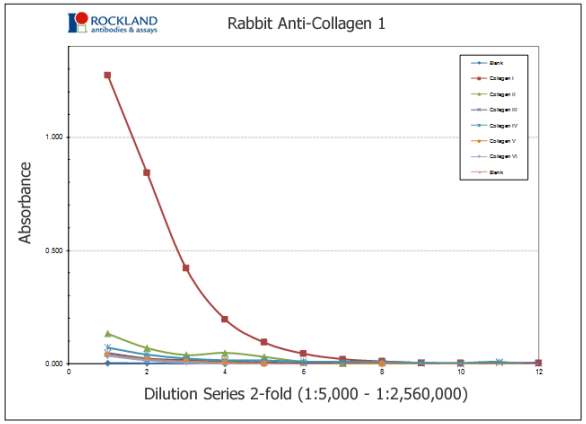
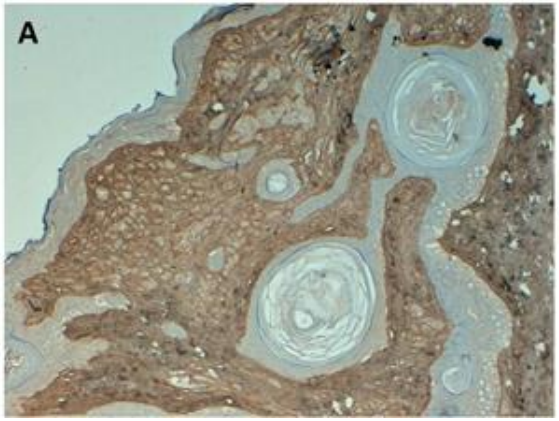
Anti-Collagen I: immunogen native non-denatured COL I.

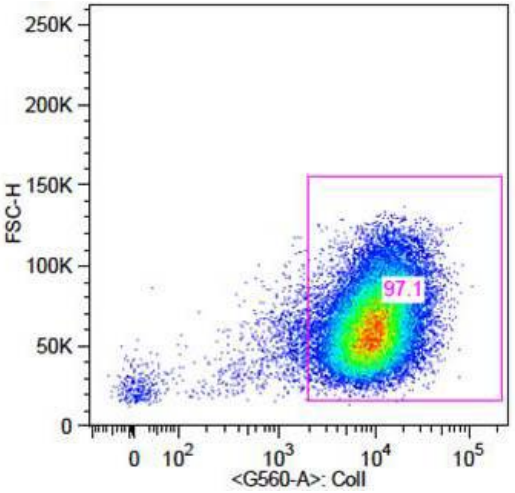
ELISA



IHC



FLOW



This antibody shows **high sensitivity** and **specificity** by ELISA, fresh frozen IHC and by FLOW – all assays that recognize native, non-denatured protein.

COLLAGEN I →

The antibody also recognizes denatured Collagen I by WB, but with a significant loss of antibody specificity.

Is this a good antibody?

#3 - Choose the Right Clonality

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Polyclonal Antibody

- Rapid. Cost efficient. Easy.
- Excellent for polyvalent antibodies.
- Multiple epitopes - good for cells.
- Preferred for precipitation reactions.
- Usually very stable in solution.
- For a shotgun approach to discovery.
- Low resource burden.
- pAbs against peptides mimic mAbs.

Monoclonal Antibody

- Highly specific for epitopes. Monovalent.
- Subcloning and screening - powerful to isolate clones with ideal properties.
- Banked hybridoma cells can supply antibody for decades.
- Can generate monoclonal antibodies to almost any substance.
- Multiple uses – for research, diagnostics and therapeutics.

Recombinant Antibody

- Defined sequence produced as recombinant molecule.
- ScFv. SdAb. VHH. Diabody.
- For diagnostic & therapeutic use?
- Phage, viral and bacterial display.
- Rab Mab recombinant proteins.
- Aptamers

#4 - Choose the Right Host

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- Host selection gives you options for:
  - The volume of antiserum required for use.
  - Intended clonality of antibody.
  - Sequence divergence from immunogen to host. Consider chicken for phylogenetic distance from mammals.
  - Pairing considerations for double and triple label experiments where secondary antibodies are planned to be used.

Flemish Giant rabbit.

Mouse, Rat, Armenian Hamster, Golden Syrian Hamster, Horse, Cow, Donkey, NZW Rabbit, Guinea Rig, Chicken, Sheep, Human.

FG Rabbit, Duck, Tobacco and other plants.

#4 - Choose the Right Host

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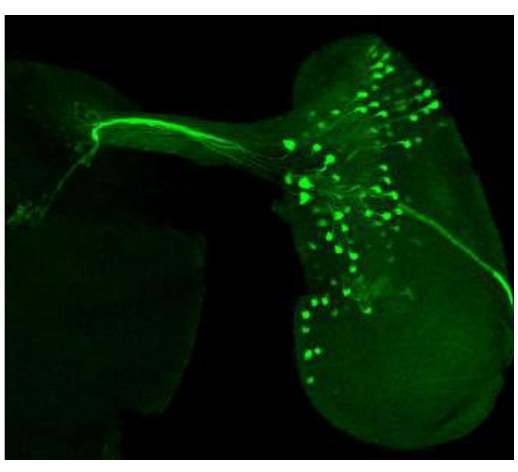
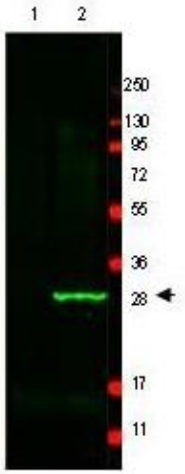
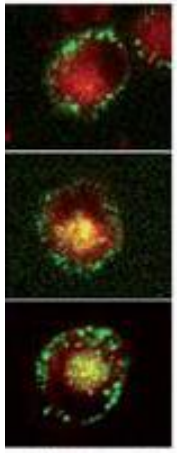
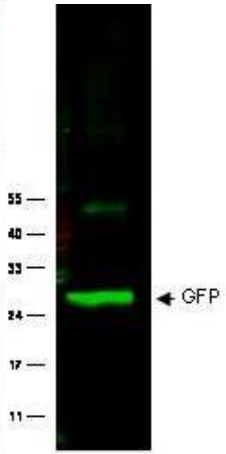
Chicken-anti-GFP



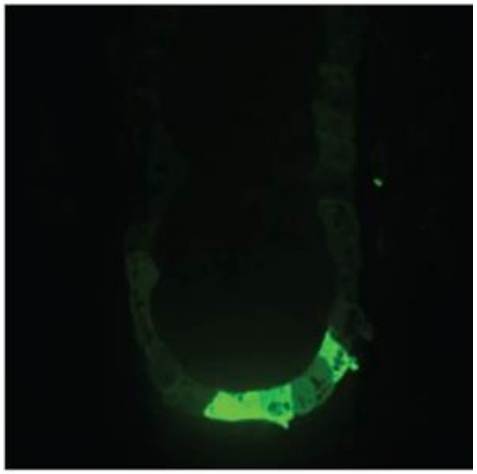


Rabbit-anti-GFP

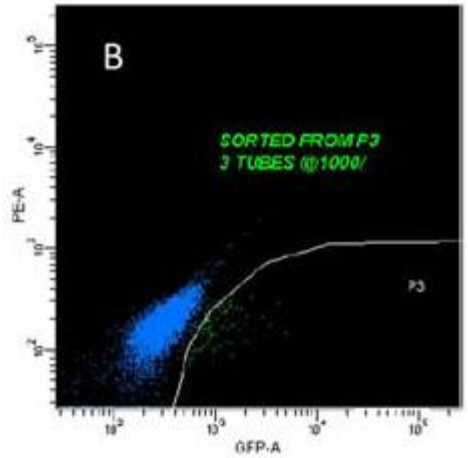




Goat-anti-GFP



Mouse-anti-GFP



#4 - Choose the Right Host

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C-C chemokine 2 [Homo sapiens] Gene CCL2 UniProtKB P13500

C-C motif chemokine 2 precursor [Oryctolagus cuniculus]

Sequence ID: ref|NP_001075763.1| **Length:** 125 **Number of Matches:** 1

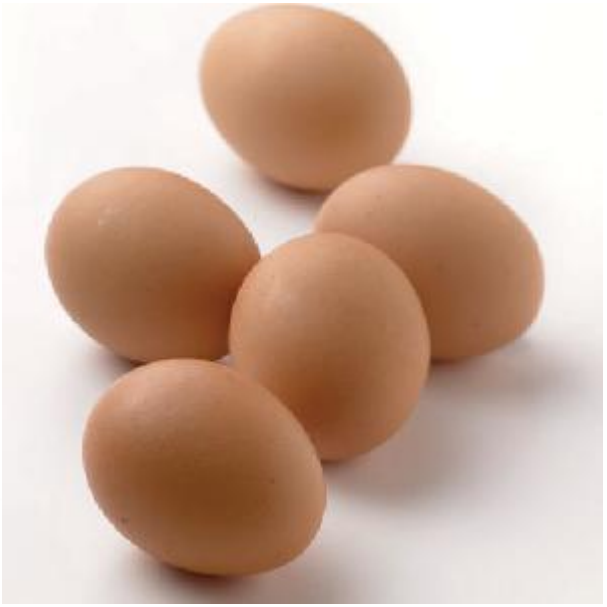
See 2 more title(s)

Range 1: 1 to 99 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
154 bits(389)	2e-45	Compositional matrix adjust.	73/99(74%)	83/99(83%)	0/99(0%)

```
Query  1   MKVSAALLCLLLIAATFIPQGLAQPDAINAPVTCCYNFTNRKISVQRLASYRRITSSKCP
           MKVSA LLCLLLIA  F    LAQPDA+N+PVTCCY FTN+ ISV+RL SYRRI S+KCP
Sbjct  1   MKVSATLLCLLLIAVAFSSHVLAQPDAVNSPVTCCYTFTNKTISVKRLMSYRRINSTKCP

Query  61  KEAVIFKTIVAKEICADPKQKWVQDSMDHLDKQTQTPKT  99
           KEAVIF T +AK ICADPKQKWVQD++ +LDK+ QTPKT
Sbjct  61  KEAVIFMTKLAKGICADPKQKWVQDAIANLDKKMQTPKT  99
```



chemokine [Gallus gallus]

Sequence ID: gb|AAD48772.1|AF146730_1 **Length:** 90 **Number of Matches:** 1

Range 1: 71 to 82 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Positives	Gaps
26.1 bits(54)	0.58	7/12(58%)	9/12(75%)	0/12(0%)

```
Query  1   ICADPKQKWVQD  12
           +CA+P   WVQD
Sbjct  71  VCANPQNDWVQD  82
```

"Avian can be a good alternative host when high sequence homology exists between the target and commonly used mammalian hosts."

#5 – PTM specific antibodies

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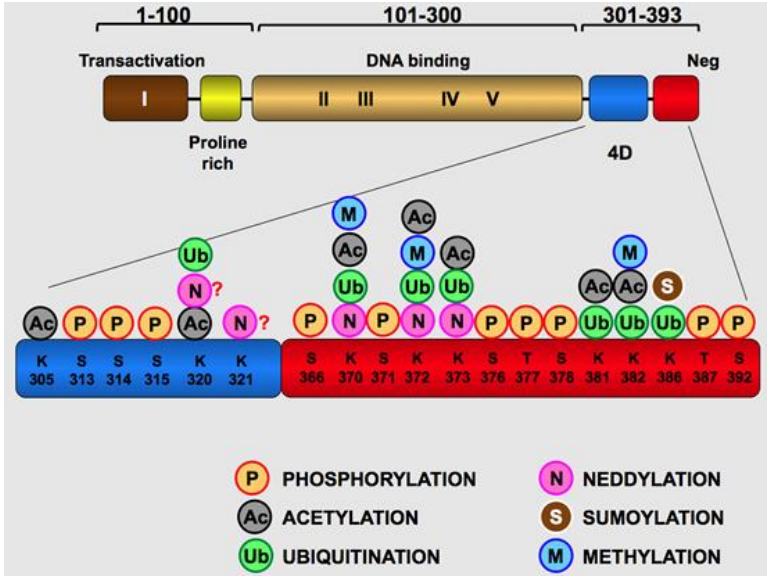
Post translational modifications for cell signaling.

- Polyclonal and monoclonal antibodies can be produced to specifically recognize PTMs.
- PTM specific antibodies can ONLY be generated using synthetic peptides.
- The modified peptide is used for immunization and for immunoaffinity chromatography.
- Unmodified peptide is for cross adsorption to render polyclonal antibodies specific for PTM.



Common PTMs include phosphorylation, acetylation, methylation, hydroxylation, glycosylation, SUMOylation, lipidation, amidylation, nitrosylation, arginylation and more.

#5 – PTM specific antibodies

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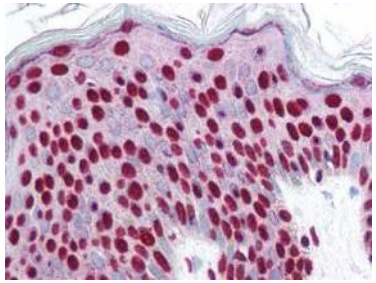
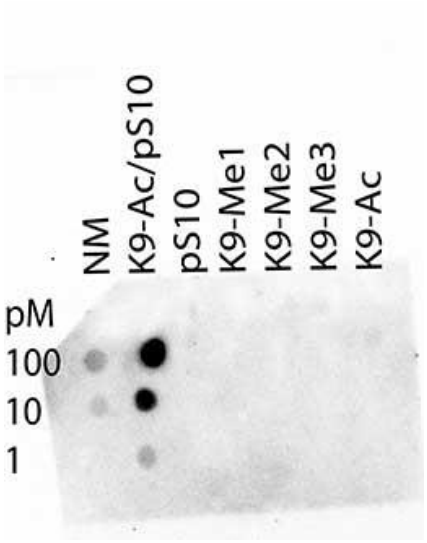
Antibodies can be made to single or dual modified sites.

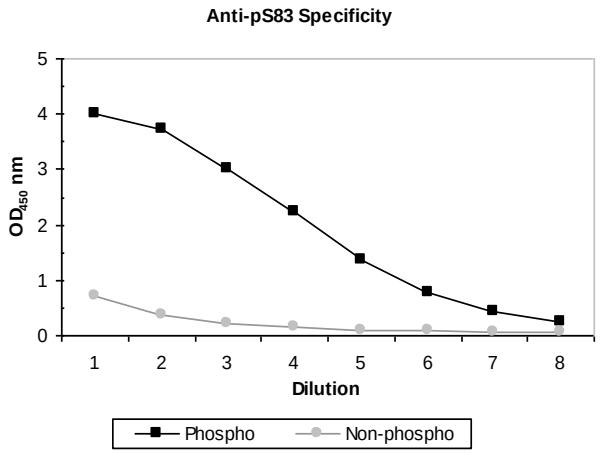
- **Anti-SMAD3 pS423/pS425**



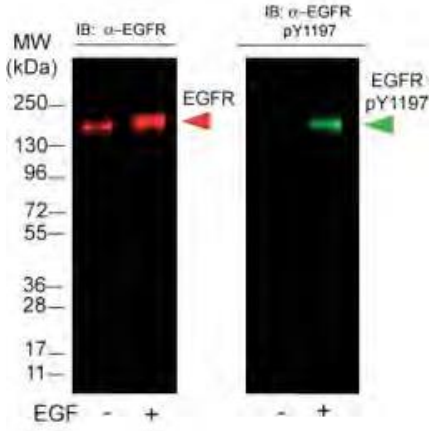
- **Anti-Histone H3 K9-Ac/pS10**



- **Anti-ASK-1 pS83**



- **Anti-EGFR pY1197**



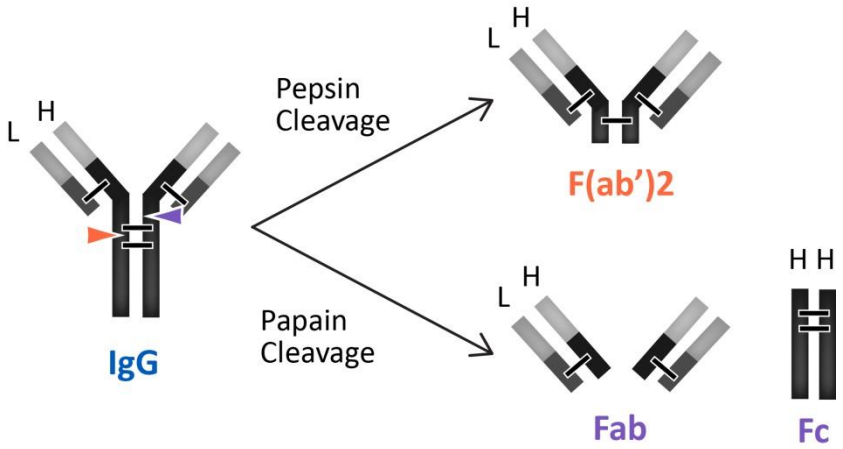
Antibodies to PTM modifications of histones are good examples of acceptable antibody specificity.

Peptides are best to produce PTM specific antibodies. Antibodies must show specificity for the surrounding sequences (context).

#6 - Antibody Fragments and Subclasses

Antibody Fragments

- $F(ab')_2$ fragments are divalent antibodies that lack F(c) mediated binding and are often used in fluorescence-activated cell sorting (FACS).
- Fab fragments are monovalent antibodies that also lack effector function. Fab fragments are reduced in size resulting in better penetration into cells and tissues for staining.



Subclasses

- When monoclonal antibodies are produced the cloned antibody can be IgM or any of the subclasses of IgG.
- Mouse IgG subclasses IgG_1, IgG_{2a}, IgG_{2b} and IgG_3 can be exploited when used in combination with subclass specific secondary antibodies for multiplexing.
- Try to avoid mouse IgM and IgG_3. Less stable. Requires higher salt levels.

% (Human)	Human	Mouse
60-65%	IgG1	IgG1
20-25%	IgG2	IgG2a
5-10%	IgG3	IgG2b
<4%	IgG4	IgG3

#6 - Antibody Fragments and Subclasses

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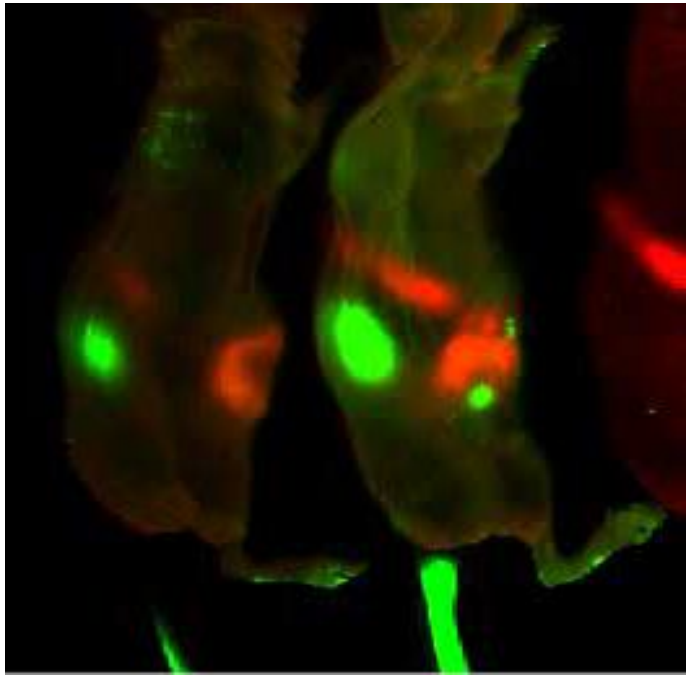
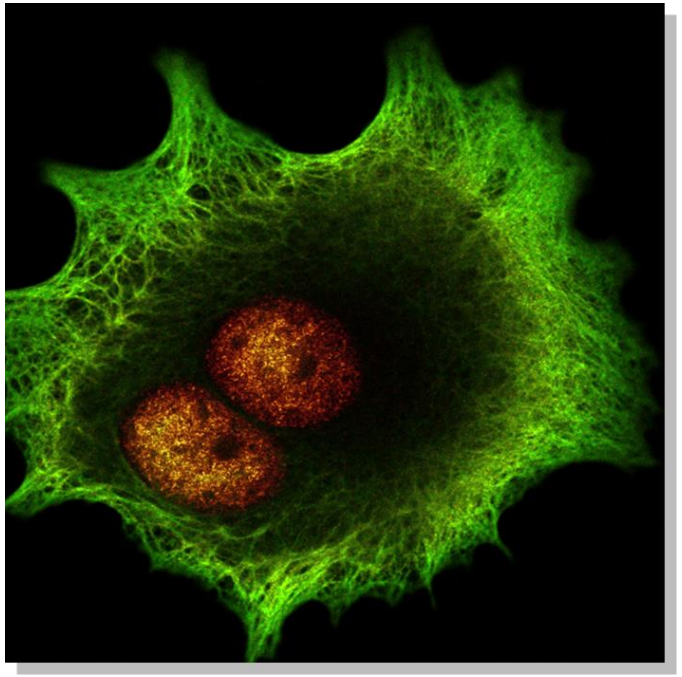
Size matters. Small molecules like Fab antibodies are best for penetrating tissues or cells and maximize resolution in microscopy.



***In vivo* imaging.**



Super resolution microscopy.

#7 – The Right Reporting System

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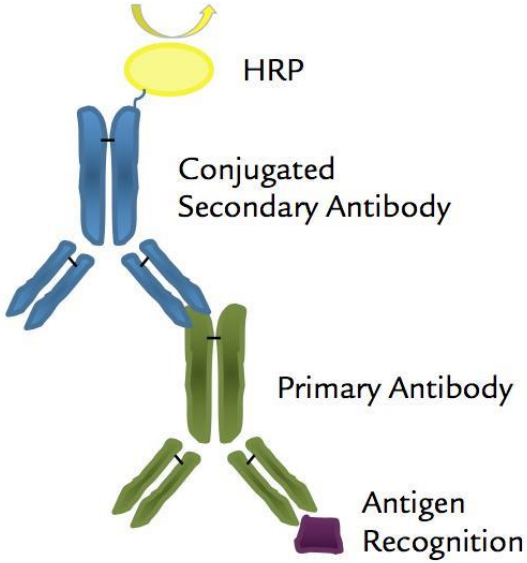
Fluorescent Label	Color	Abs (nm)	Em (nm)	MW (daltons)
DyLight™ 405	Blue	400	420	793
ATTO 425	Blue	436	484	498
Cy2™	Blue Green	489	505	897
DyLight™ 488	Blue Green	493	518	1,011
ATTO 488	Green	501	523	981
Fluorescein (FITC)	Green	495	528	390
ATTO 532	Yellow Green	532	553	1081
Cy3™	Yellow Green	552	565	949
DyLight™ 549	Yellow Green	550	568	982
Rhodamine (TRITC)	Orange	550	570	444
R-Phycoerythrin (RPE)	Orange	488	575	240,000
ATTO 550	Orange	554	576	791
Cy3.5™	Orange Red	581	596	1,286
Texas Red®	Red	596	620	625
ATTO 594	Red	601	627	1389
Allophycocyanin	Far-Red	650	660	100,000
Cy5™	Far-Red	650	667	975
ATTO 647N	Far-Red	644	669	843
DyLight™ 649	Far-Red	646	674	1,008
ATTO 655	Far-Red	663	684	887
Cy5.5™	Near Infra-Red	678	703	1,312
DyLight™ 680	Near Infra-Red	682	715	950
DyLight™ 800	Infra-Red	770	794	1,050

- All antibodies require systems to detect the binding of antigens. Furthermore, quantitative immunoassays must generate data that can be measured and correlated with either antigen or antibody levels.
- For Western blot and ELISA, an enzyme conjugated secondary used in combination with a substrate is the best choice. Commonly used enzyme reporters included Peroxidase or Alkaline Phosphatase can act on colorimetric and luminescent substrates.



- Fluorescent assays like FLISA, immunofluorescence microscopy and flow cytometry require fluorescent reporters (i.e. FITC, DyLight™, ATTO-tec, Alexafluor or Cy™ dye).
- Be mindful of the linear range of the detection assay and the relative concentration of analyte in the sample to be tested.

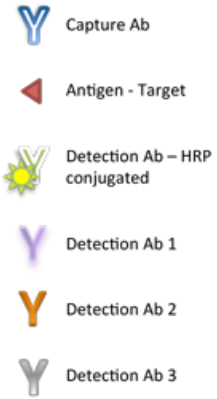
#8 - Limits of Specificity and Sensitivity

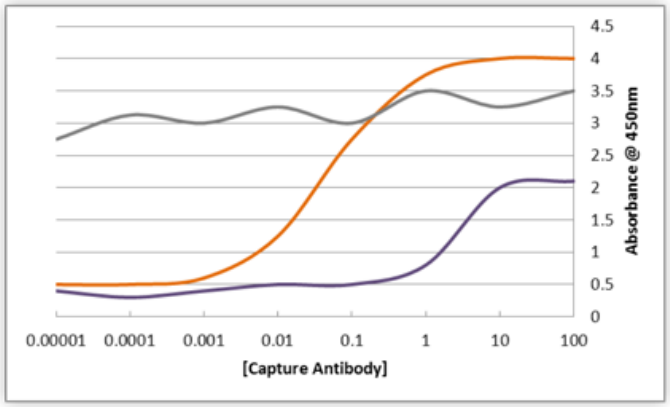
Specificity - qualitative

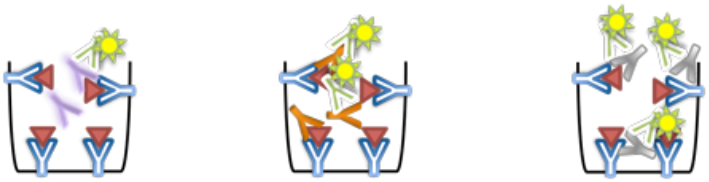
- Specificity refers to the properties of an antibody to bind to one or more antigens. It is a qualitative measurement.

Sensitivity - quantitative

- Sensitivity refers to how much antibody is needed to elicit a reaction. It is a quantitative measurement.







High specificity. Low sensitivity.

High specificity. High sensitivity.

Low specificity. High sensitivity.

#9 - Consider Antibody Lifespan

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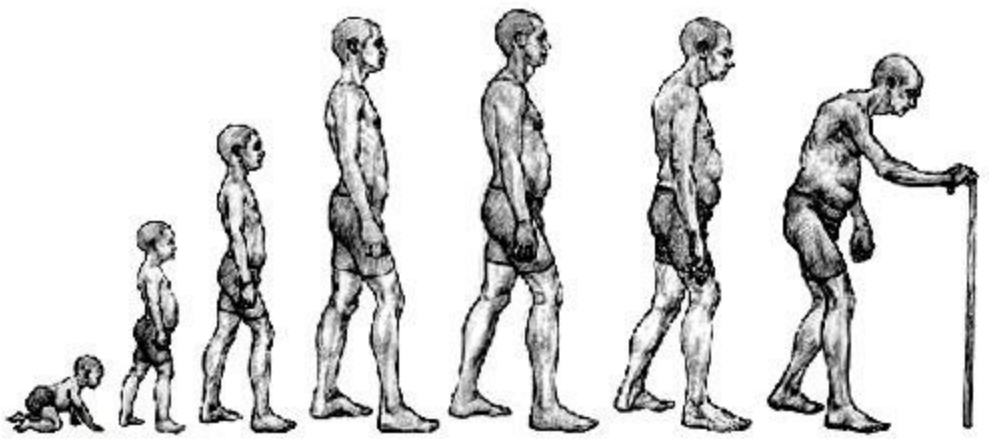

- You are a **graduate student**. Just one serum collection from one rabbit gives you 20 mL of high titer antiserum that you use at 1:1,000 for WB. You now have 20L of antibody at 1X. Using 15 mL per WB you can run over 1,300 WBs. Life is good. Write your dissertation.

- You are a **PI** and must provide a critical antibody to 20 scientists in your lab. Your buddy at UT and the UCSF wants some too. Immunize a goat or sheep for higher yields. Consider a pool from multiple rabbits. If a mAb consider a large roller bottle run.

- You are a **diagnostic company** and the FDA just gave final approval of your assay. A polyclonal antibody is a critical reagent and you need a 10 year supply to provide the US and Europe with 10,000 units per year. Consider large cohorts for polyclonal antibodies with 3-5L of antiserum as reserves. If a mAb consider a bioreactor run. You will need a bridging study to prove equivalence between multiple lots.

You may or may not be able to reproduce the EXACT properties of an antibody if you run out.

#10 - Benefits of Antibody Pools

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Pooling can enhance the specificity of polyvalent antibodies.

- Don't be afraid to pool antibodies to obtain a desired specificity.
- For pAbs pools of antiserum can be created after preliminary screening and before final purification.
- In a multiple cohort study, you can pool based on different collection dates from the same host, or the same collection date from multiple hosts.
- Only pool antibodies from the same host to avoid elevated background staining.

Antibody Basics

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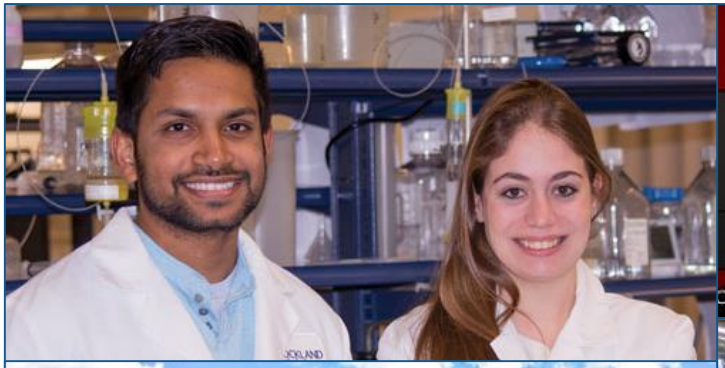
1. Pick the right immunogen for the job.
2. Know your intended assay before you start.
3. Choose the right clonality for your purpose.
4. Choose the right host to control for sequence divergence.
5. Know what it takes to generate a PTM specific antibody.
6. Understand how antibody fragments and subclasses affect outcomes.
7. Know what reporting system is the right choice for your work.
8. Understand limits on specificity and sensitivity.
9. Consider antibody lifespan when you generate an antibody.
10. Understand the benefit of antibody pools.

For More Information

ROCKLAND
antibodies & assays



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