Immunohistochemistry: Role in biotechnology-derived pharmaceutical regulatory studies

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Genentech
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Agenda

- Regulatory Background
- Applications:
  - Tissue Cross-Reactivity Studies
- Issues and Challenges

Why do Health Authorities care?

Identification of potential off-target, cross-reactive epitopes.

Monoclonal antibodies administered to humans have usually been well tolerated. Instances of serious or fatal adverse events have generally resulted from intended or unintended binding of MAb to specific epitopes.

Non-target tissue binding could have serious consequences.

ICH S6(R1)* Species Selection: General Principles

- Comparison of target sequence homology
- *In vitro* qualitative and quantitative cross-species comparison of relative target binding affinities, receptor/ligand occupancy and kinetics
- Assessments of functional activity
  - *In vitro* – species-specific cell-based systems
  - *In vivo* pharmacology or toxicology studies
  - Modulation of a known biologic response or of a pharmacodynamic marker
- Capability to demonstrate potentially adverse consequences of target modulation (consideration of species differences in target binding and functional activity)
- **Assessment of tissue cross reactivity in animal tissues is of limited value**; however, in specific cases TCR can be used to guide selection by comparison of profiles in human and those animals tissues where target binding is expected.

*Pre-clinical Safety Evaluation of Biotechnology Derived Pharmaceuticals, June 2011

The Extracellular Domain of Monkey* ErbB2 Is Highly Homologous to Human

- Sequence identity is 99% (644/650)
- Amino acid differences occur at:

-M177V, Q377R, F404L, H552R, A601T, P604S

```
Human   STQVCTGTDMKLRLPASPETHLDMLRHLYQGCQVVQGNLELTYLPTNASLSFLQDIQEVQ  60
Monkey  ------------------------------------------------------------

Human   GYVLIAHNQVRQVPLQRLRIVRGTQLFEDNYALAVLDNGDPLNNTTPVTGASPGGLRELQ  120
Monkey  ------------------------------------------------------------

Human   LRSLTEILKGGVLIQRNPQLCYQDTILWKDIFHKNNQLALTLIDTNRSRACHPCSPMCKG  180
Monkey  ------------------------------------------------------V-----

Human   SRCWGESSEDCQSLTRTVCAGGCARCKGPLPTDCCHEQCAAGCTGPKHSDCLACLHFNHS  240
Monkey  ------------------------------------------------------------

Human   GICELHCPALVTYNTDTFESMPNPEGRYTFGASCVTACPYNYLSTDVGSCTLVCPLHNQE  300
Monkey  ------------------------------------------------------------

Human   VTAEDGTQRCEKCSKPCARVCYGLGMEHLREVRAVTSANIQEFAGCKKIFGSLAFLPESF  360
Monkey  ------------------------------------------------------------

Human   DGDPASNTAPLQPEQLQVFETLEEITGYLYISAWPDSLPDLSVFQNLQVIRGRILHNGAY  420
Monkey  ----------------R--------------------------L----------------

Human   SLTLQGLGISWLGLRSLRELGSGLALIHHNTHLCFVHTVPWDQLFRNPHQALLHTANRPE  480
Monkey  --------------------------------R---------------------------

Human   DECVGEGLACHQLCARGHCWGPGPTQCVNCSQFLRGQECVEECRVLQGLPREYVNARHCL  540
Monkey  ------------------------------------------------------------

Human   PCHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVARCPSGVKPDLSYMPIWKFPDEEG  600
Monkey  ------------------------------------------------------------

Human   ACQPCPINCTHSCVDLDDKGCPAEQRASP
Monkey  T--S------------------------
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Scatchard Analysis of Herceptin Binding

Human 650 ± 85 pM
Monkey 719 ± 15 pM

Scatchard Analysis of Pertuzumab Binding

Human 800 ± 80 pM
Monkey 530 ± 70 pM

**Cynomolgus*

ICH S9 Nonclinical Evaluation for Anticancer Pharmaceuticals (*patients with advanced cancer*)

- Minimal considerations for initial clinical trials in patients…disease refractory or resistant to available therapy…
  - Important to clearly define the patient population, supports extent of nonclinical studies required even after approval of initial indication

- Clarification of guidance provided by Question and Answer document:
  - **Tissue cross reactivity studies generally have little utility and are not needed unless there is a specific cause for concern**
  - A scientific assessment for toxicity to reverse should be provided but recovery groups are not automatically expected
  - Supportive care during toxicology studies may be appropriate
  - Alternative *in vitro* and *in vivo* assays can be used to aid in the assessment of reproductive risks
  - Dose-range finding study can be used to show clear evidence of embryo-fetal lethality or teratogenicity

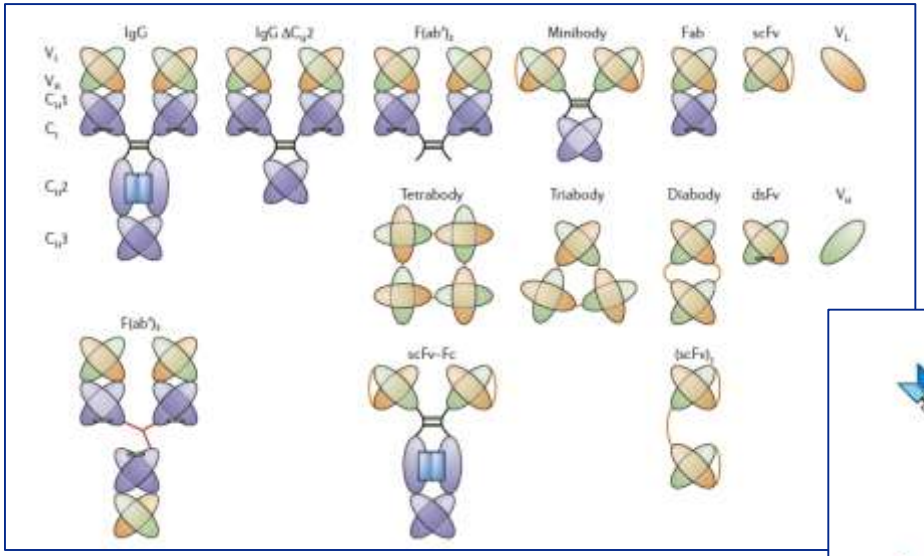
March 2010 (FDA); Q&A April 2018

Regulatory Immunohistochemistry Caveats/Constraints

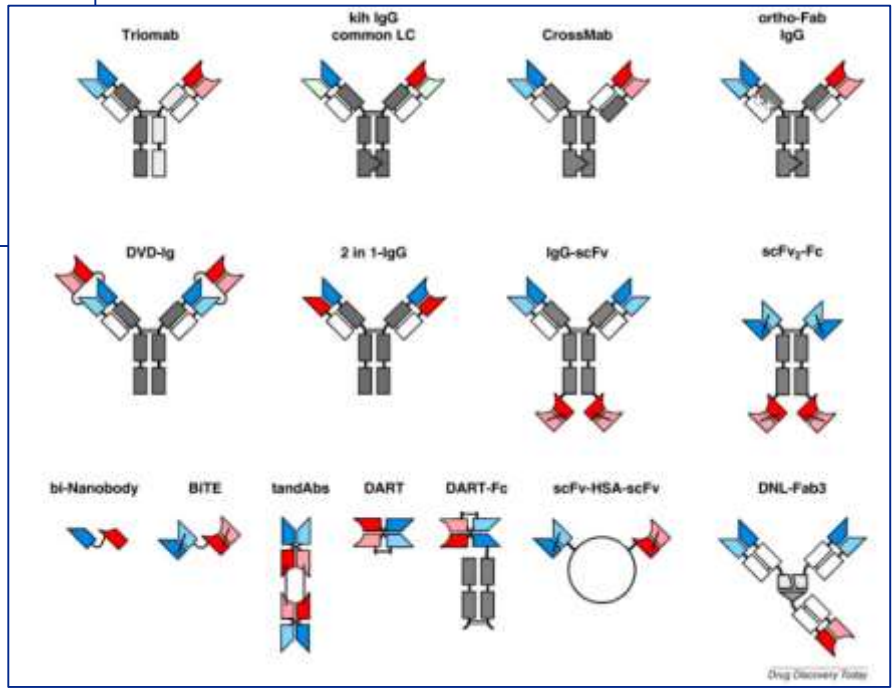
- Study objective to rule out unexpected epitope/"off-target" binding, not to describe intended target distribution.
- Therapeutic antibody rather than optimal IHC antibody reagent
  - Use form of test article to be used clinically (eg conjugates, antibody fragments) for studies
- "Quick-frozen" adult tissues
- Test several concentrations of the product
- Positive and negative controls

FDA, PTC, 1997

Representative mono- and bispecific therapeutic antibody formats

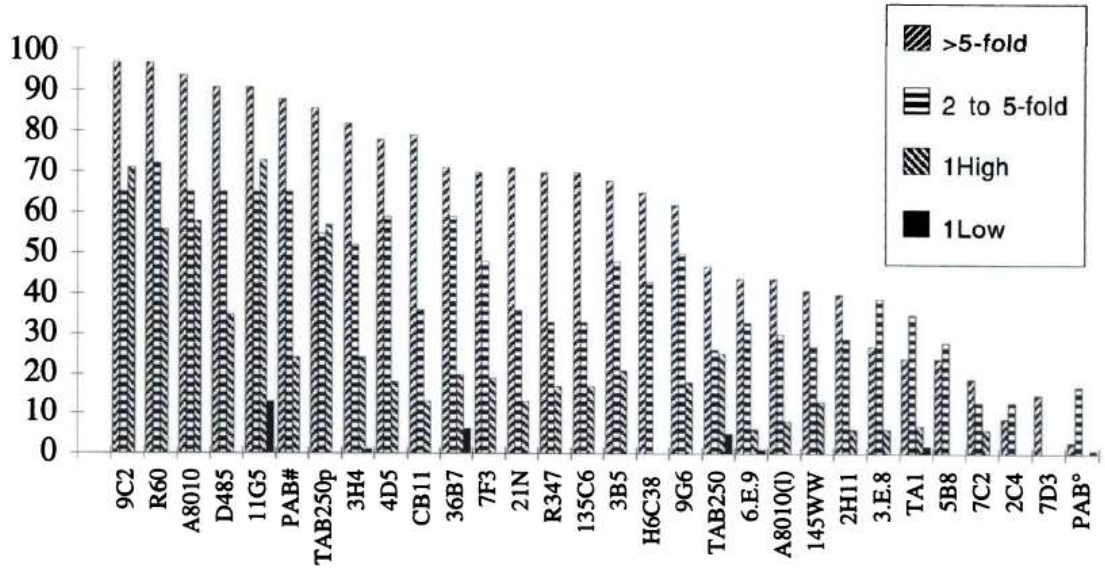


Carter P, Nature Rev Immunol 6:343, 2006



Kontermann R, Brinkmann U
Drug Disco Today 20:838, 2015

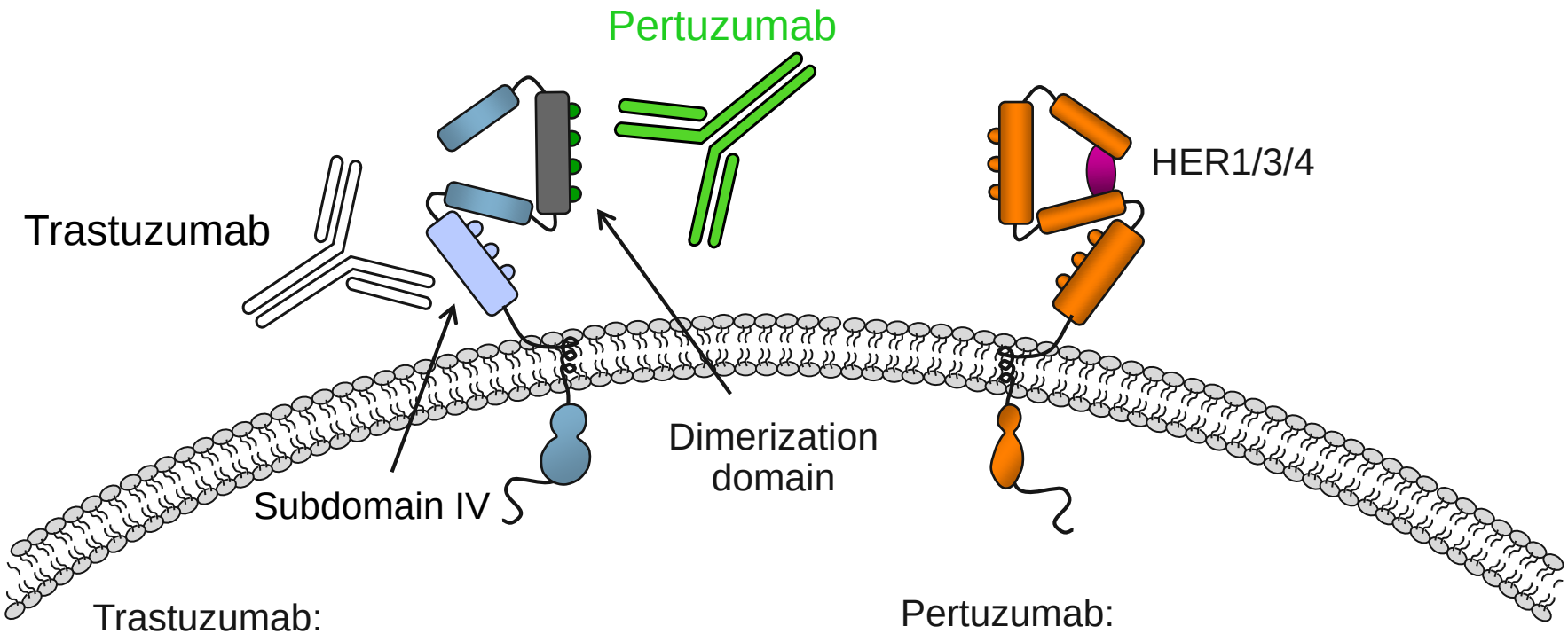
Antibody comparative IHC performance



Antibody	Breast Cancer immunostained (%)	Sensitivity (%)	Specificity (%)
“Ideal” result	37	100	100
R60 (polyclonal)	29	80	100
4D5	21	57	100
2C4	3	8	100

Press MF, et al Canc Res, 54:2771, 1994

Pertuzumab and Trastuzumab Bind Different Sites on the HER2 Extracellular Domain



Trastuzumab:

- Inhibits ligand-independent HER2 signaling
- Activates ADCC
- Prevents HER2 ECD shedding

Pertuzumab:

- Inhibits ligand-dependent HER2 dimerization and signaling
- Activates ADCC

ADCC, antibody-dependent cell-mediated cytotoxicity; ECD, extracellular domain

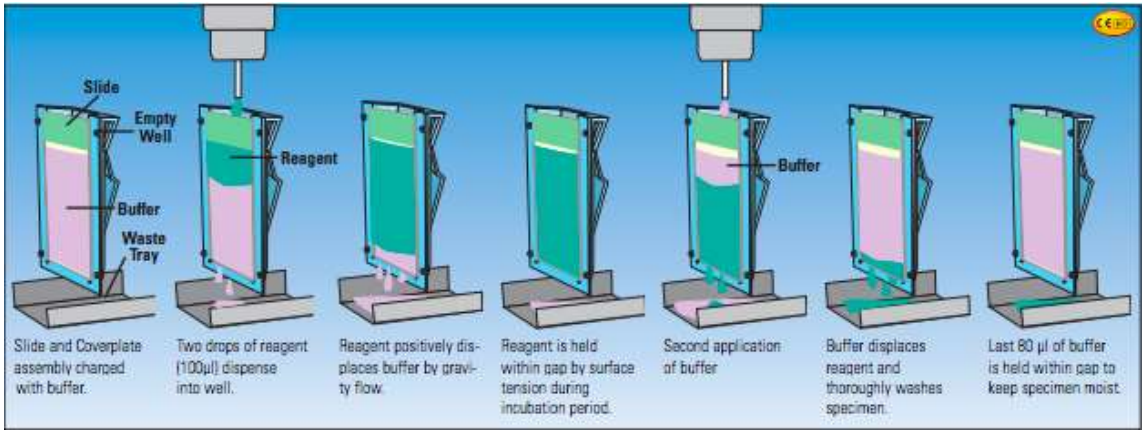
HER-2 Human Tissue Expression

Tissues	Press et al 1990*	Herceptin TCR	Perjeta TCR
Skin	+	+	+
Gastrointestinal epithelium	+	+	Neg
Salivary gland	+	+	Neg
Pancreas	+	+	Neg
Respiratory - bronchi	+	+	Neg
Reproductive	+	+	+
Breast	+	+	+
Urinary Bladder	+	+	+
Heart	Neg	Neg	Neg
“unexpected”	Neg	Neg	Neg

* Tissue expression confirmed by Northern, Southern, Western and IHC

Press MF, et al, Oncogene, 5:593, 1990

Capillary Gap IHC: Shandon™ Sequenza™



https://www.fishersci.ie/shop/products/thermo-scientific-shandon-sequenza-immunostaining-center-2/10098889

http://cellab.se/pdfbroschyr/inkubation/Sequenza%20Immunostaining%20Center.pdf

Capillary Gap IHC

- Pros
  - Supports large batch runs
  - Adaptable to variety of methods
  - Accurate timing
  - Effective buffer washes
  - Coverplate system protects sections
  - 100 ul reagent/slide
- Cons
  - Uneven staining
    - Bubbles – reagents must be at room temp; mount slide to coverplate as though coverslipping
    - Multiple sections per slide – unless offset can result in uneven flow of reagents
  - Consumable expense
    - Coverplate reuse
    - Microdamage during cleaning

GLP Tissue Cross Reactivity studies utilize frozen tissues

Attributes

- Antigen/epitope unaffected by crosslinking fixatives
- Tests antibody specificity and sensitivity
- Widely available
- Rapid procedure
- Light microscope based
- Antigen detection with morphology/histology intact
- No matrix dilution

Challenges

- Tissue quality and consistency
  - Artefact common
- Stability of antigen/epitope
- Variable antigen density in tissue section
- Resolution – morphology can be indistinct
- Antibody variability in suitability (for use as IHC reagent)

Quick-frozen adult tissues

- Surgical samples preferred
- Extensive list provided
- Tissues from 3 unrelated human donors

PTC, FDA, 1997

Quick-frozen “surgical samples preferred”

- Adrenal
- Bladder
- Bone Marrow
- Breast
- Cerebellum
- Cerebral Cortex
- Colon
- Endothelium
- Eye
- Fallopian Tube
- Gastrointestinal Tract
- Heart
- Kidney (glomerulus, tubule)
- Liver
- Lung
- Lymph Node
- Ovary
- Pancreas
- Parathyroid
- Pituitary
- Prostate
- Skin
- Spinal Cord
- Spleen
- Striated muscle
- Testis
- Thymus
- Thyroid
- Ureter
- Uterus (cervix, endometrium)

Post-mortem samples the routine

Post-mortem samples are the routine

Limitations:

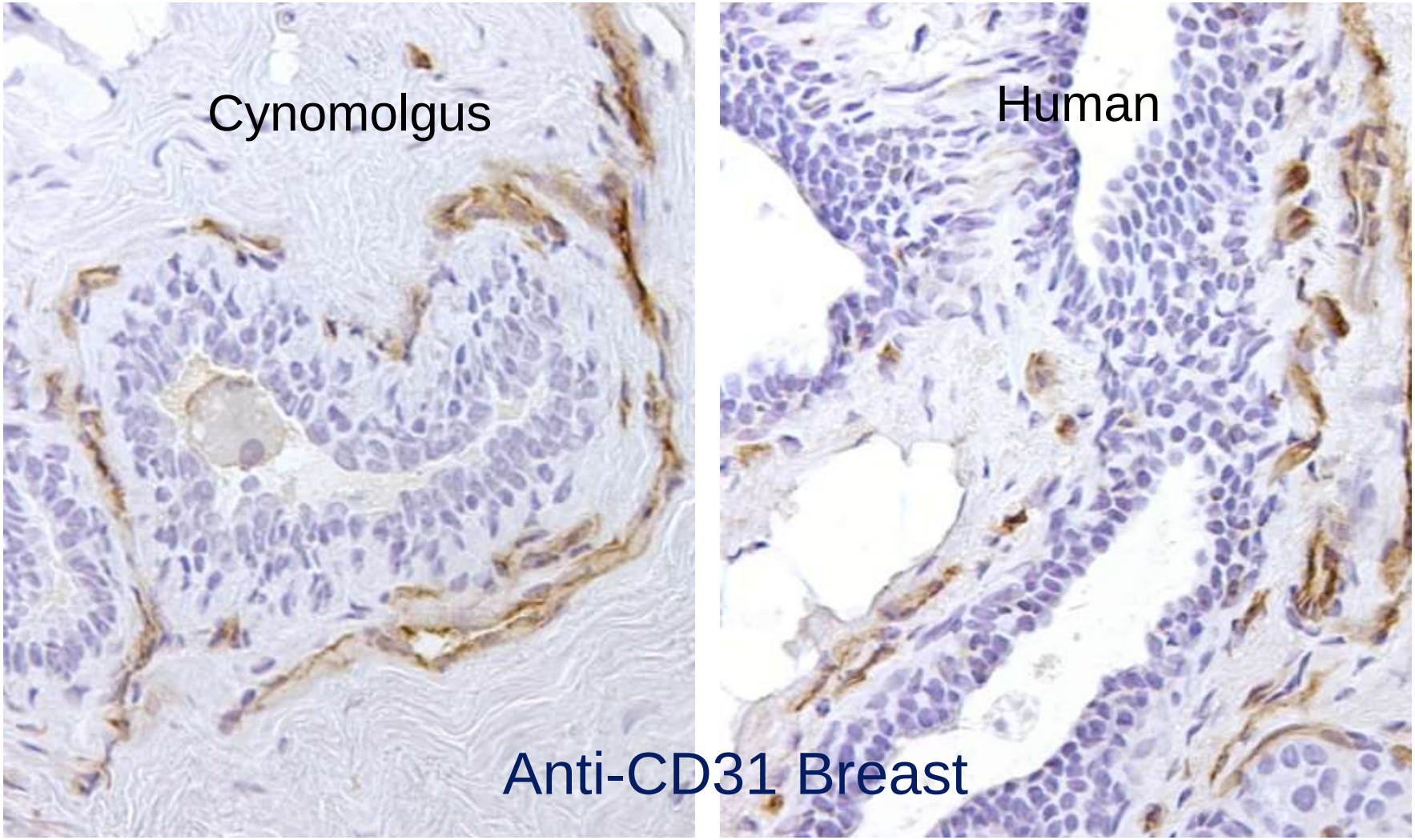
Postmortem interval – variable

Challenge: Epitope stability

Solution: Quality control test tissue with ‘housekeeping’ antibody such as CD31 or, ß2 microglobin

Drawback: Antigen of interest may be more “delicate” than the ‘housekeeping’ protein.

Tissue Quality Controls



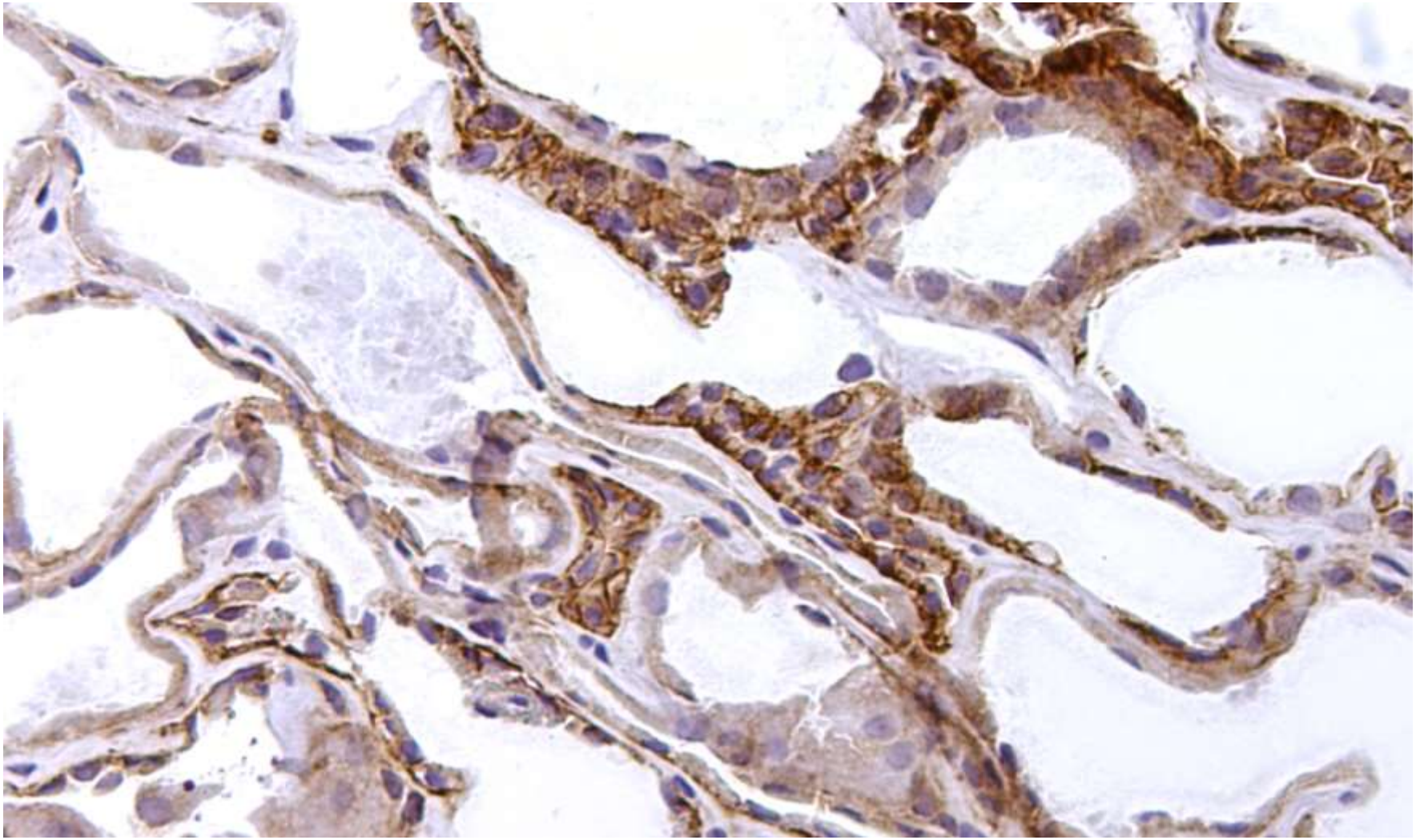
Post-mortem samples are the routine

Limitations:

- Postmortem interval – variable
  - Poor morphology

- "Quick frozen"
  - Common Protocol = OCT on dry ice or, in liquid Nitrogen
  - Freezing is too slow leads to less than optimal preservation of morphology (and antigens)

Tissue Quality Limitations



IHC for Detection of Protein on Frozen Tissues

Attributes

- Antigen/epitope unaffected by crosslinking fixatives
- “Tests” antibody specificity and sensitivity
- Widely available
- Rapid procedure
- Light microscope based
- Antigen detection with morphology/histology intact
- No matrix dilution

Challenges

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Staining Procedure

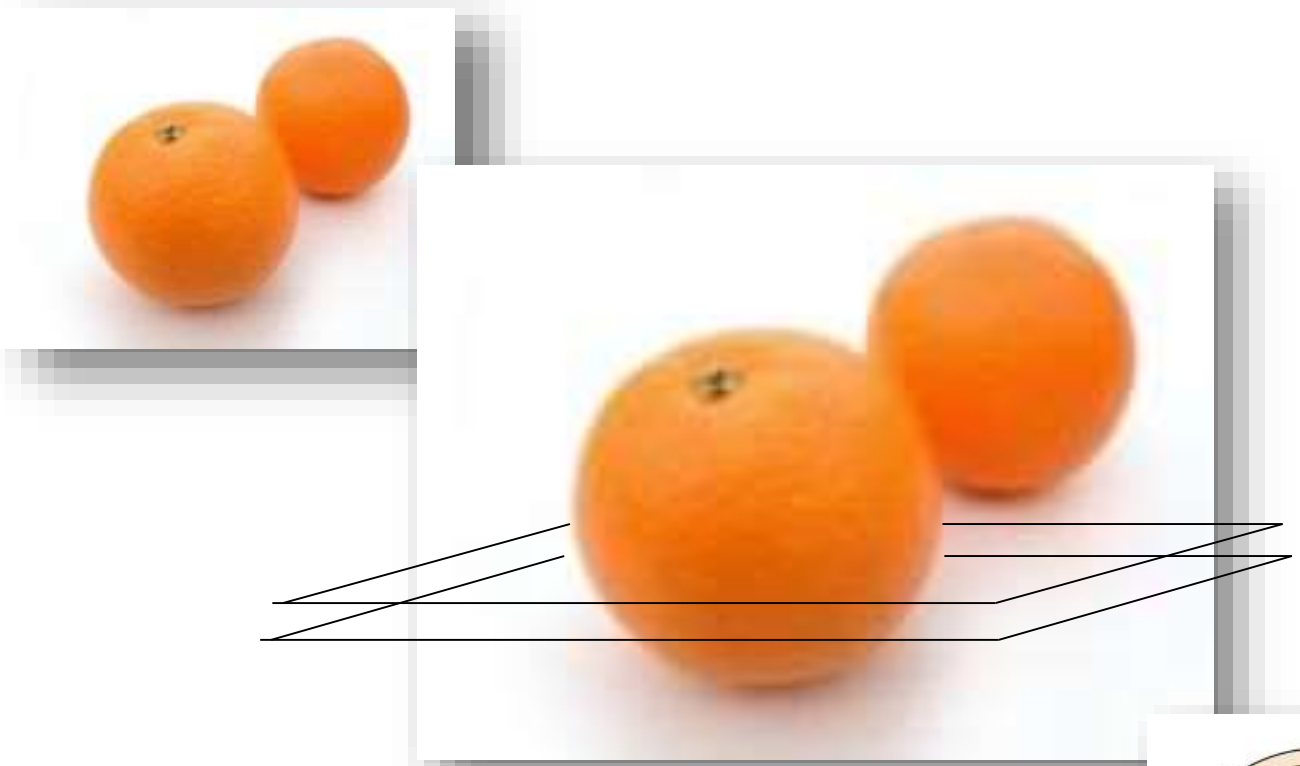
1. Blocking step – reduce non-specific staining
2. Primary antibody (therapeutic Mab or BspAb)
3. Next steps; dependent on Step 2:
   - Direct: Labeled primary MAb/BspAb (depends on label, eg. Biotin or, FITC)
   - “Cocktail” Indirect: Unlabeled primary MAb pre-incubated with labeled secondary prior to application to tissue section
   - Traditional Indirect

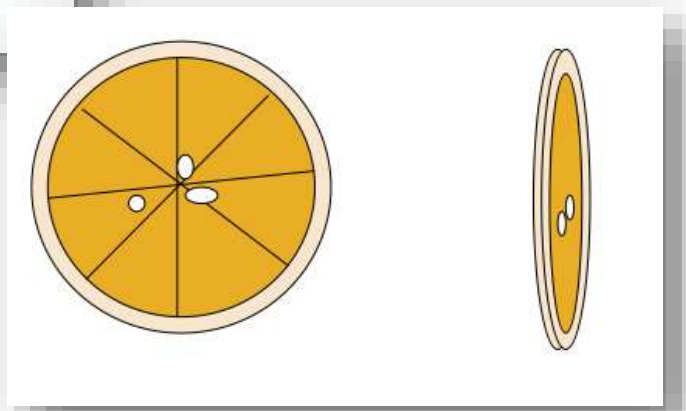
Test several concentrations of the product

- Ideal or, optimum concentration generally obtained by titration analysis
  - Lowest concentration that produces maximum binding to the target antigen (FDA, 1997)

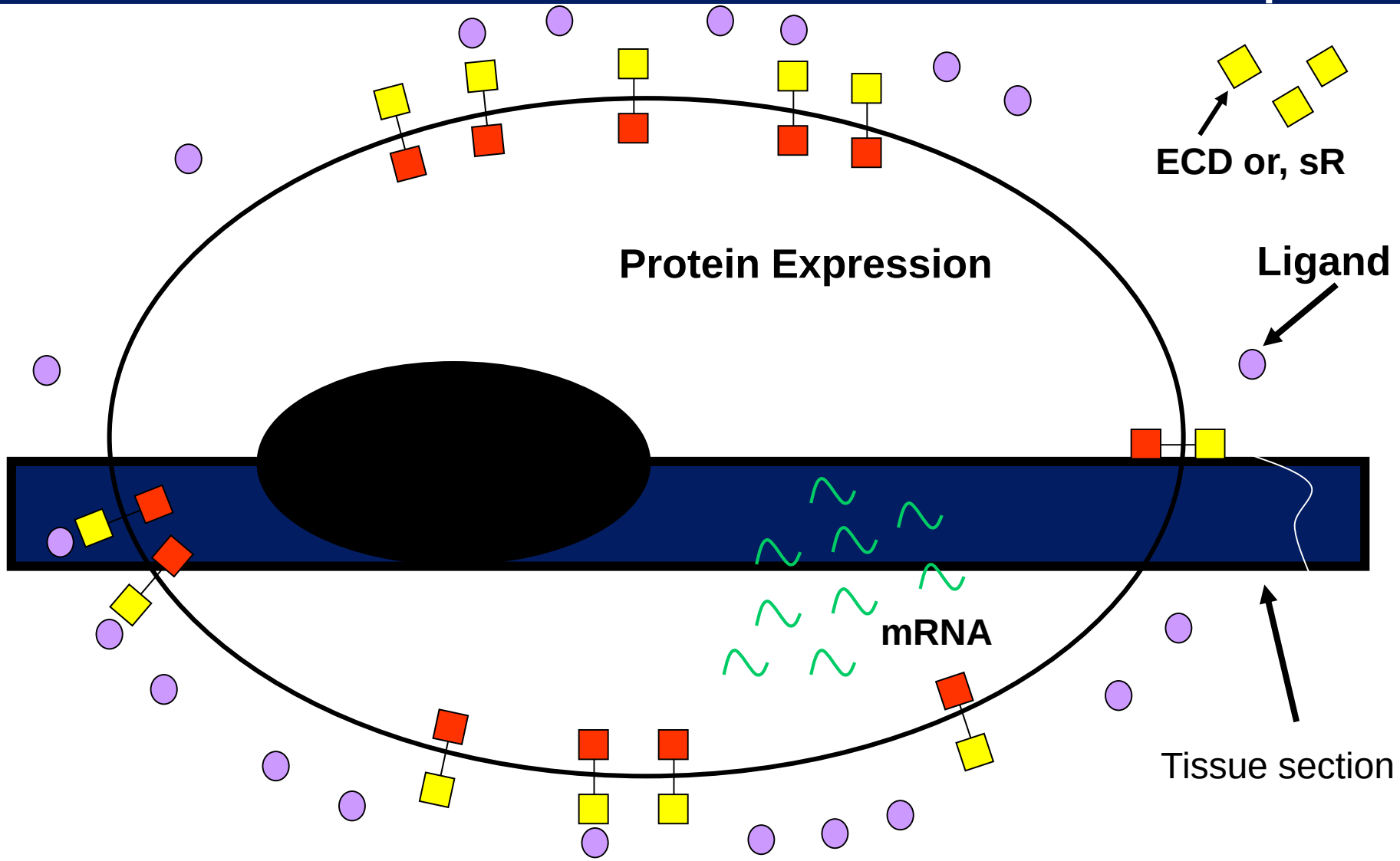
- “Consideration of peak plasma concentration”
  - Reality: second antibody dilution evaluated is 5 to 10 fold increase over optimal concentration

Cells *in vivo* versus tissue section

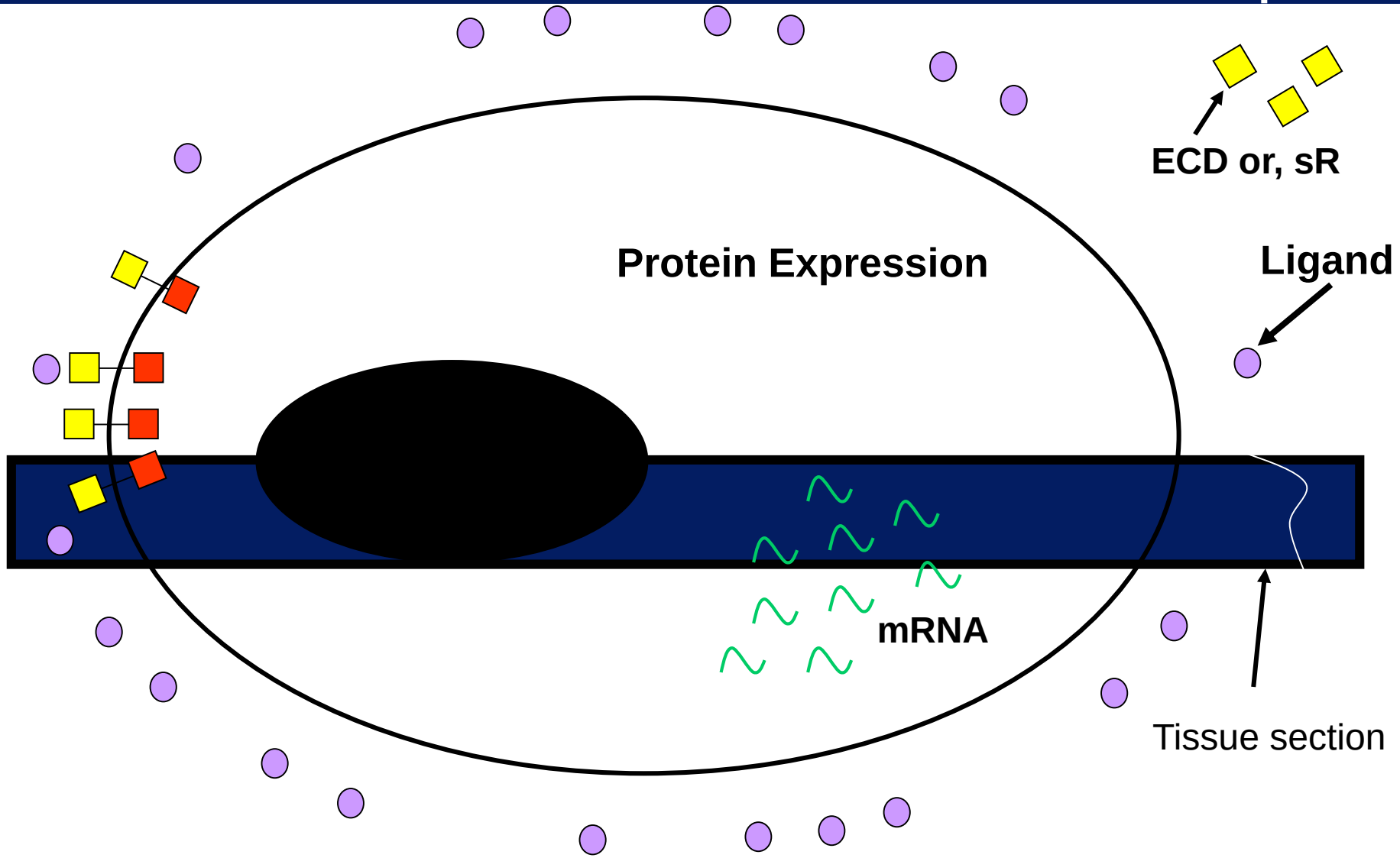




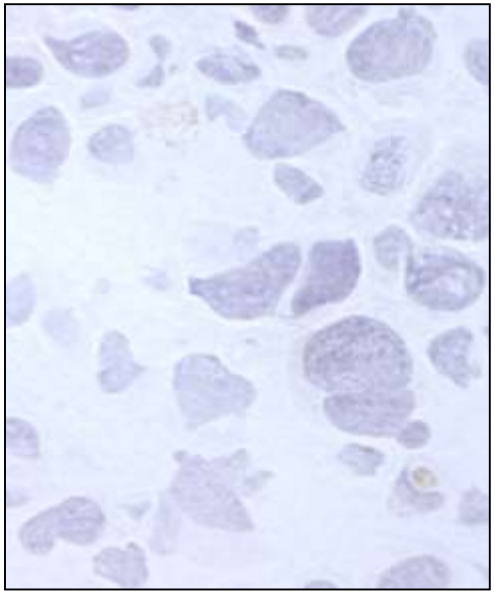
Moderate” Protein Expression



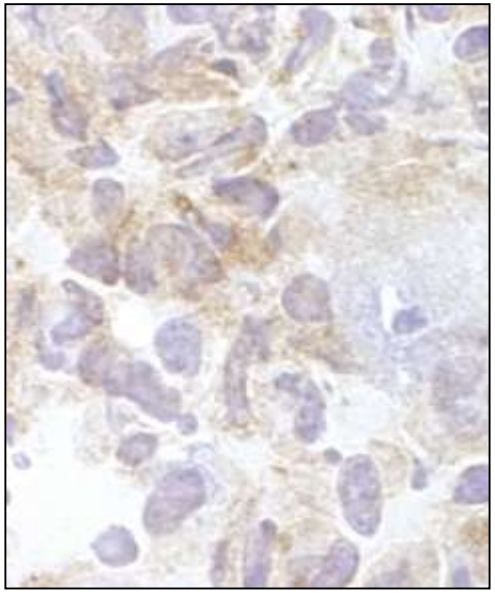
Sparse Localized Protein Expression



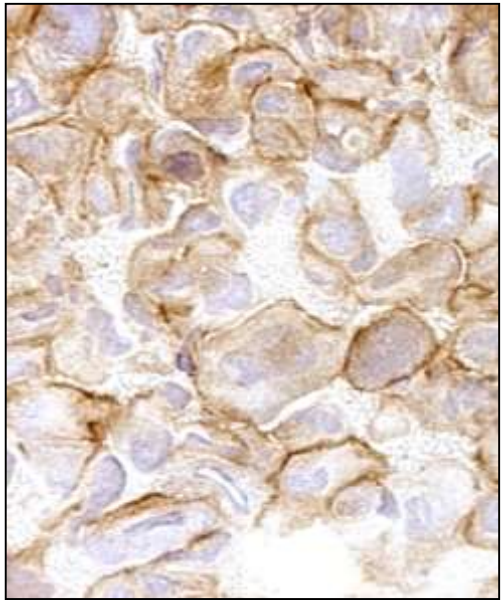
Antigen/Epitope Expression Controls



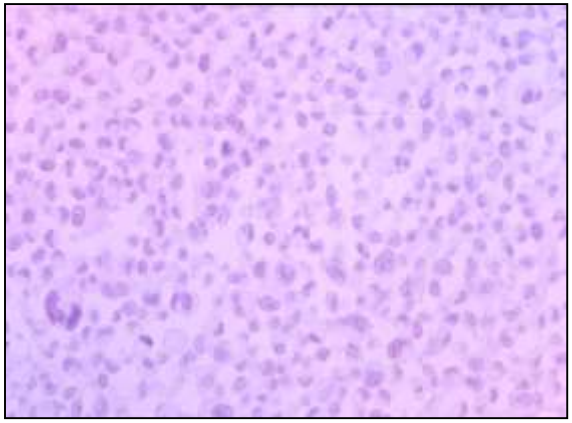
20,000/cell

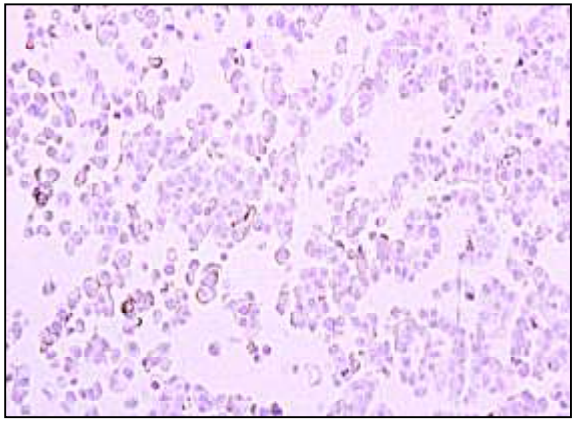


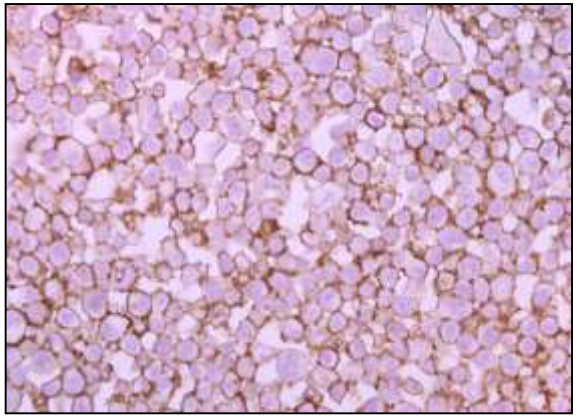
100,000/cell



1-2 x10^6/cell





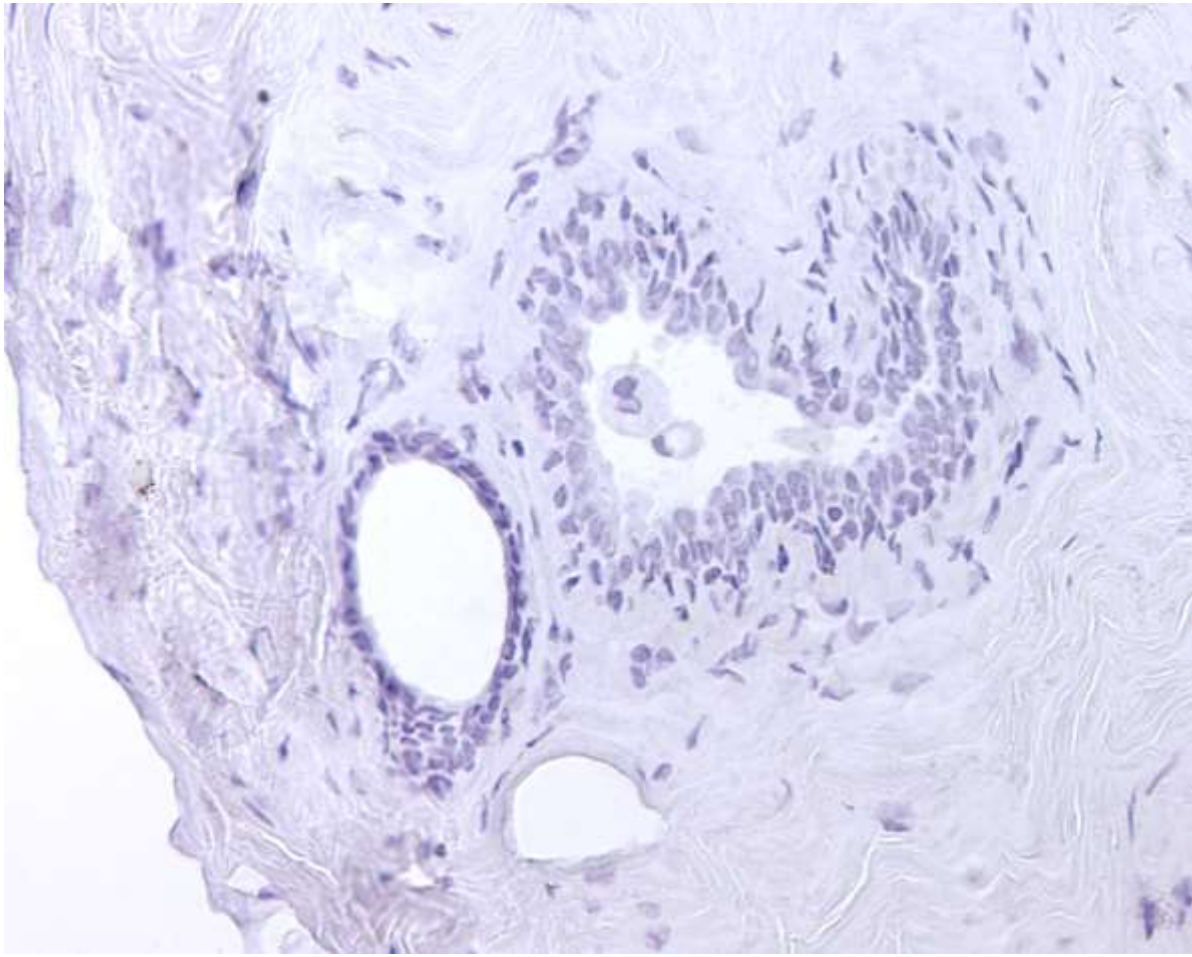


Positive and negative controls

- Tissue Controls: confirm acceptable condition of the tissues and adequacy of the assay
  - Probe for known antigen (eg CD31, ß2 microglobin, transferrin receptor, etc) to evaluate condition of protein in tissue to be used for cross reactivity screen
- Postive and negative control tissues
  - Tissues and/or cell lines characterized for presence and absence of antigen
- Assay/Reagent controls
  - Isotype control = ‘irrelevant’ (ie non-specific binding) antibody of same isotype as MAb
  - Omission of primary MAb to test for non-specific binding of secondary antibody (indirect method) or, reaction of detection reagents.

Assay/Reagent Control

Isotype Control
Human Breast



Microscopic evaluation and scoring

- Confirm tissue quality
- Confirm appropriate control staining
- Score staining based on intensity, frequency and cellular location
- Evaluate specific versus non-specific staining

IHC for Detection of Protein: requirements

- Quality tissue sections
- Expert method optimization
- Expert reagent development
- Understanding of limitations

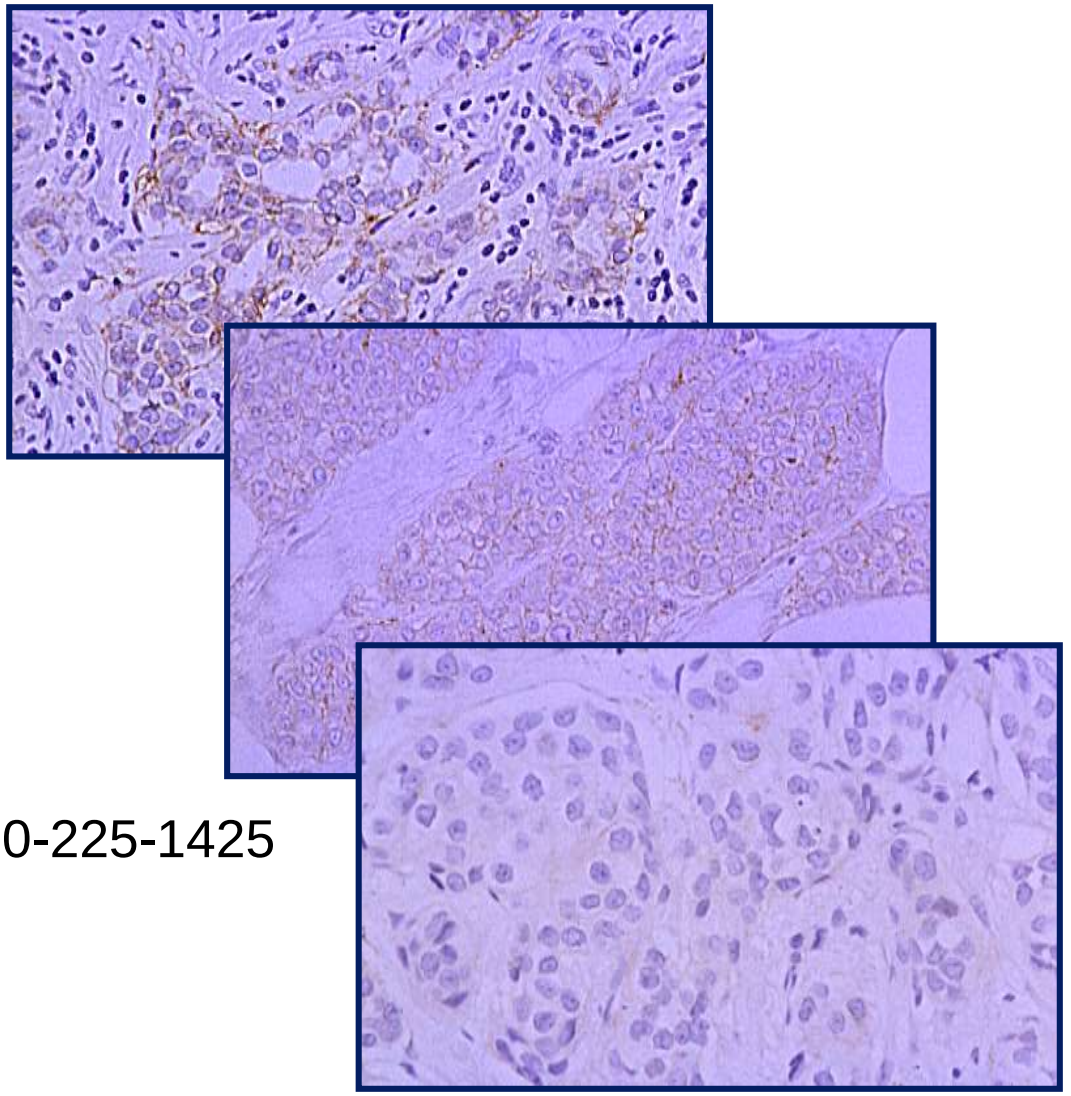
Summary

- ICH S6(R1) and S9 provide guidance on application of TCR
  - Identification of off-target epitopes
- Poor sensitivity of therapeutic antibodies as reagents can result in a high rate of false negatives
- Difficulties with control tissue quality and IHC method can result in moderate to high rate of false positives
- Safety species selection can be supported by but should not be based on TCR results

Thank you!

- Audience!

- gRED
  - Wendy Halpern
  - Linda Rangell
- CRO partners
  - CRL – Reno
  - Covance – Harrogate



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