# Laboratory 10

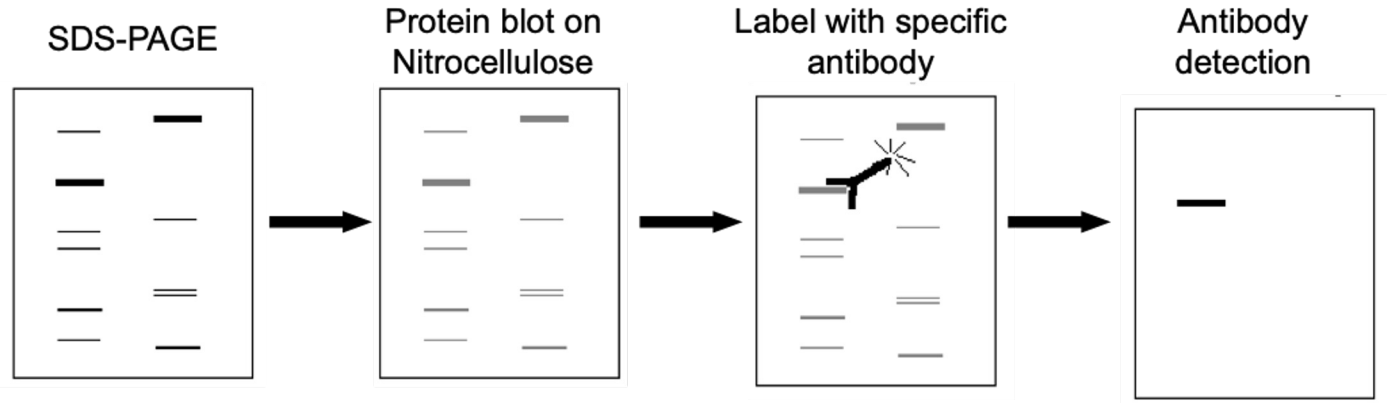


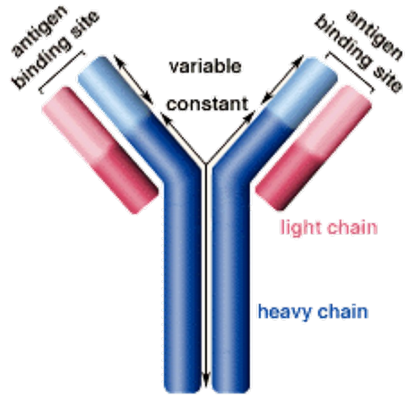
Immunoblot

Enzyme assay

# Immunoblot




SDS-PAGE
Protein blot on Nitrocellulose
Label with specific antibody
Antibody detection


antigen binding site
variable
constant
antigen binding site
light chain
heavy chain

# Experimental steps



## Blocking (nitrocellulose membrane)

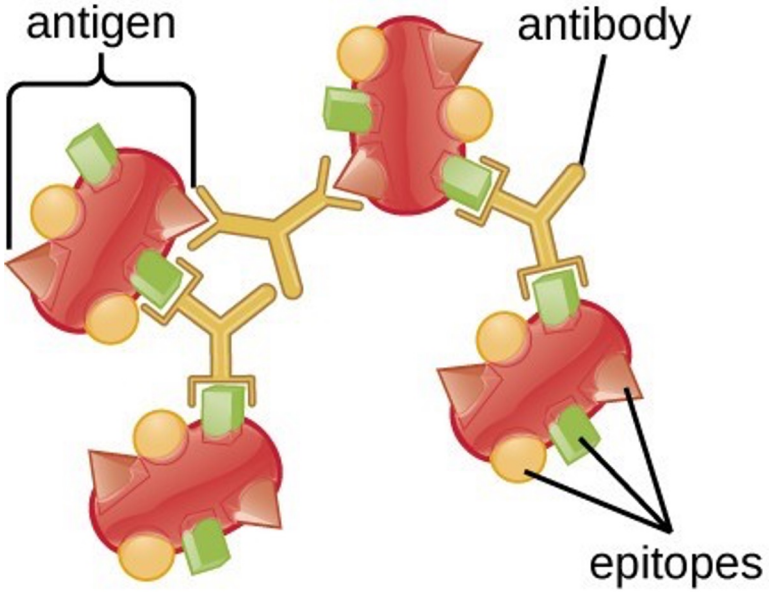
- Reduces unspecific binding (less background)
- PBS-Tween buffer + BSA or skim milk

## Primary Antibody binding

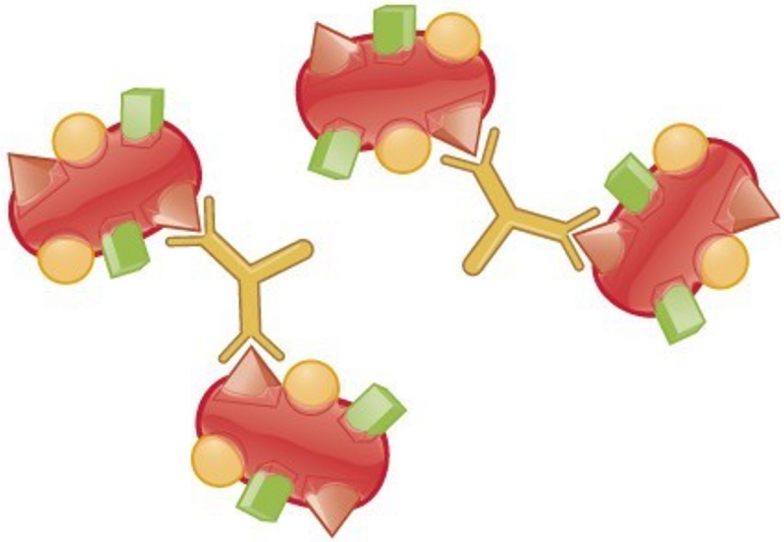
- Antibody specifically binds to protein of interest
- Specificity depends on the antibody

# Polyclonal versus Monoclonal Antibodies

Polyclonal antiserum




Monoclonal antibodies



# Polyclonal antibodies



Recognizes **multiple epitopes** - signal amplification

Serum obtained from antigen injected animals will contain a heterogeneous complex mixture of antibodies of different affinity

Production is inexpensive and fast

Batch to batch variability possible

# Monoclonal antibodies



Recognize only **one epitope**

High specificity and reproducibility

Production is expensive and long (hybridoma)

Sequence must be known and not patent protected (recombinant antibody)

# Example Primary and Secondary Antibody

## α-Amylase

- **Primary antibody:** polyclonal anti-amylase antibody from goat
- **Secondary antibody:** anti-goat IgG (from donkey)

## Actin

- **Primary antibody:** recombinant monoclonal anti-actin antibody rabbit IgG
- **Secondary antibody:** anti-rabbit IgG (from donkey)

# Detection AMY2 Fusion Protein



We will take advantage of the Myc and 6xHIS tag present at C-terminus of recombinant AMY2 and use anti-Myc-HRP monoclonal antibody
This semester we will test different concentrations of recombinant antibodies

Do these antibodies recognize porcine amylase?

# Antibody detection

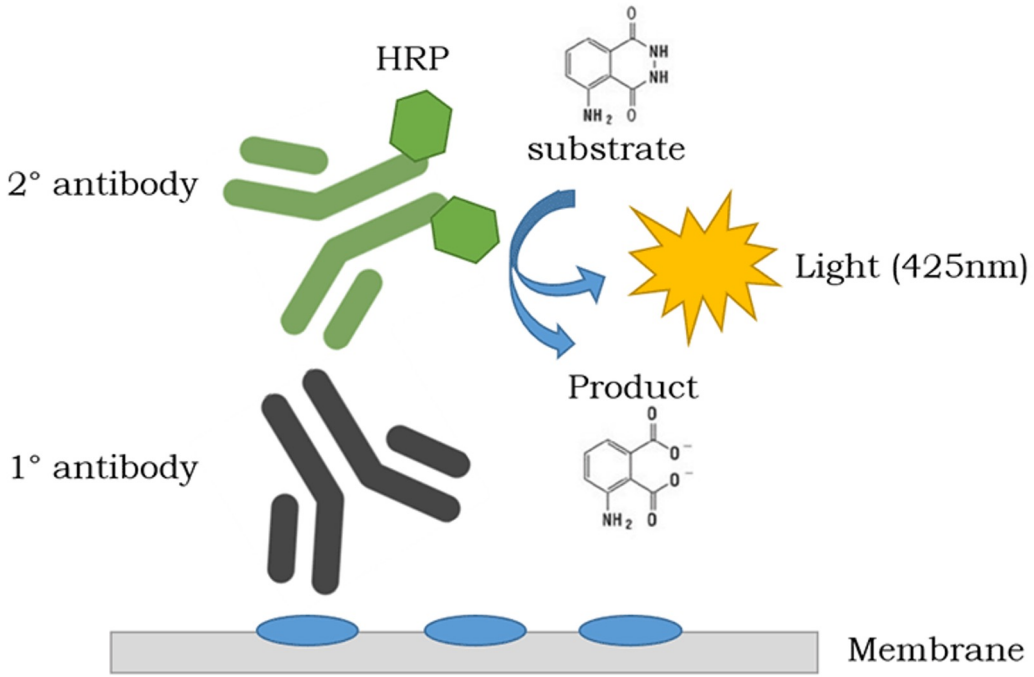


Methods to detect secondary antibody:

- **Chemiluminescence: Antibody coupled to Horse Radish Peroxidase (HRP)**
- Colorimetric
- Fluorescence
- Radioactive

# Chemiluminescent Detection






- Enzyme (HRP) linked to **secondary antibody** cleaves chemiluminescent agent.
- Emitted light is detected using imaging system.

Source: chemiluminescent-western-blotting

# Incubation with substrate for enhanced chemiluminescence (ECL)

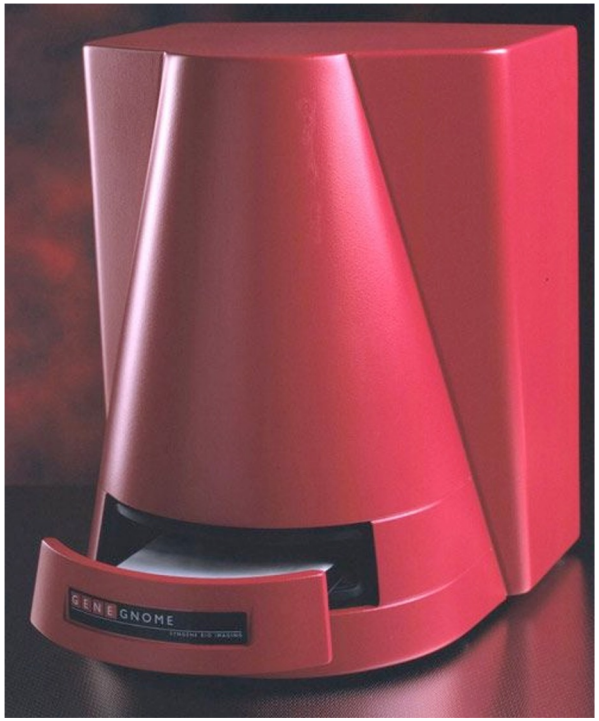
Add the substrate mix when you are at the GeneGnome imaging system

Enzymatic reaction: wait 5’ before taking the image

The membrane must not dry out (enzymes!)

The protein side of the blot must face the camera for light detection

# Chemiluminescent Imaging System: GeneGnome




We will take one image (cooled high resolution CCD camera) of the prestained marker and one after incubation with the substrate.

**Settings**

Marker: white light; 1 second exposure
Chemiluminescence: no light; 1-5 minutes exposure

# Amylase enzyme activity assay



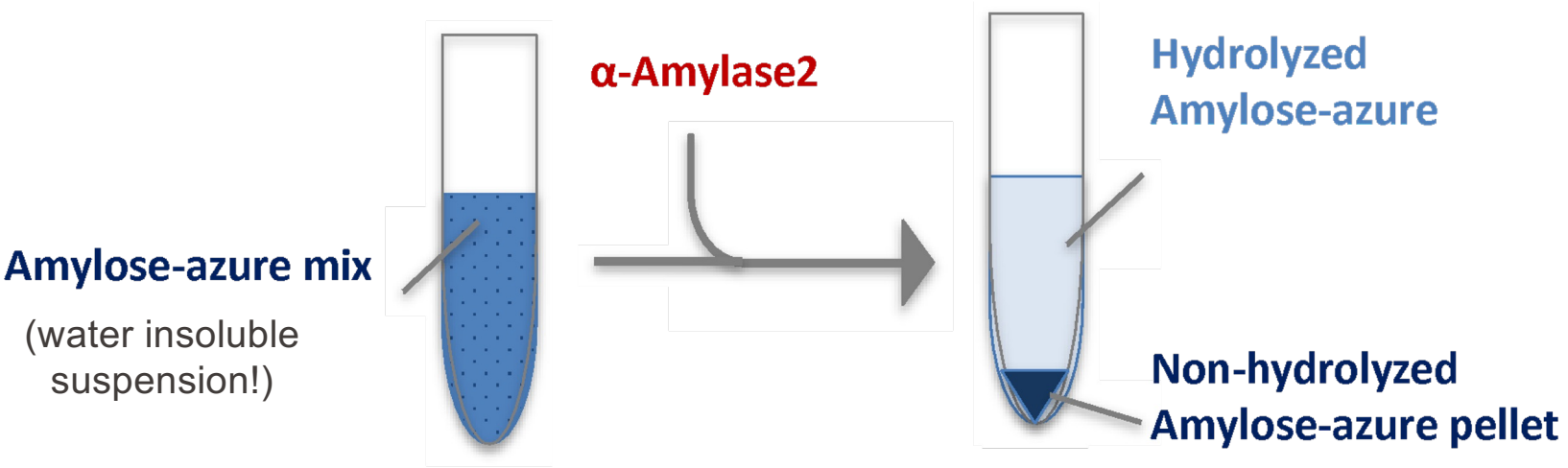
Amylases are glycoside hydrolases and act on α-(1-4)-glycosidic bonds.

They degrade starch, glycogen and other polysaccharides into glucose and maltose.

Amylose-azure is a linear polysaccharide linked to a blue dye (water-insoluble).

TASK: Measure amylase activity in test samples and controls

# Amylase enzyme activity assay

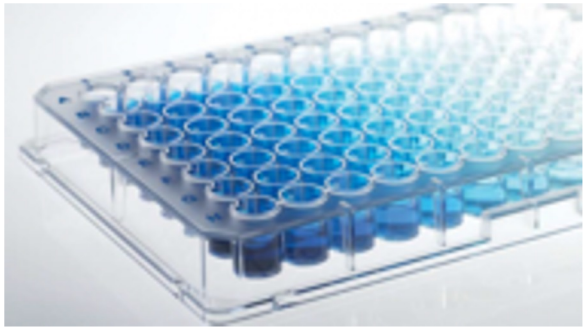



To set up the reaction:
Vortex Amylose-azure before pipetting!

After the reaction:
spin down and transfer only supernatant (hydrolyzed amylose-azure) to 96-well plate!

# Amylase assay





Measure absorbance of hydrolysed amylose-azure at 595nm

In case you transfer accidentally insoluble blue substrate (visible by eye, high absorbance measurement), remove such wells from the analysis