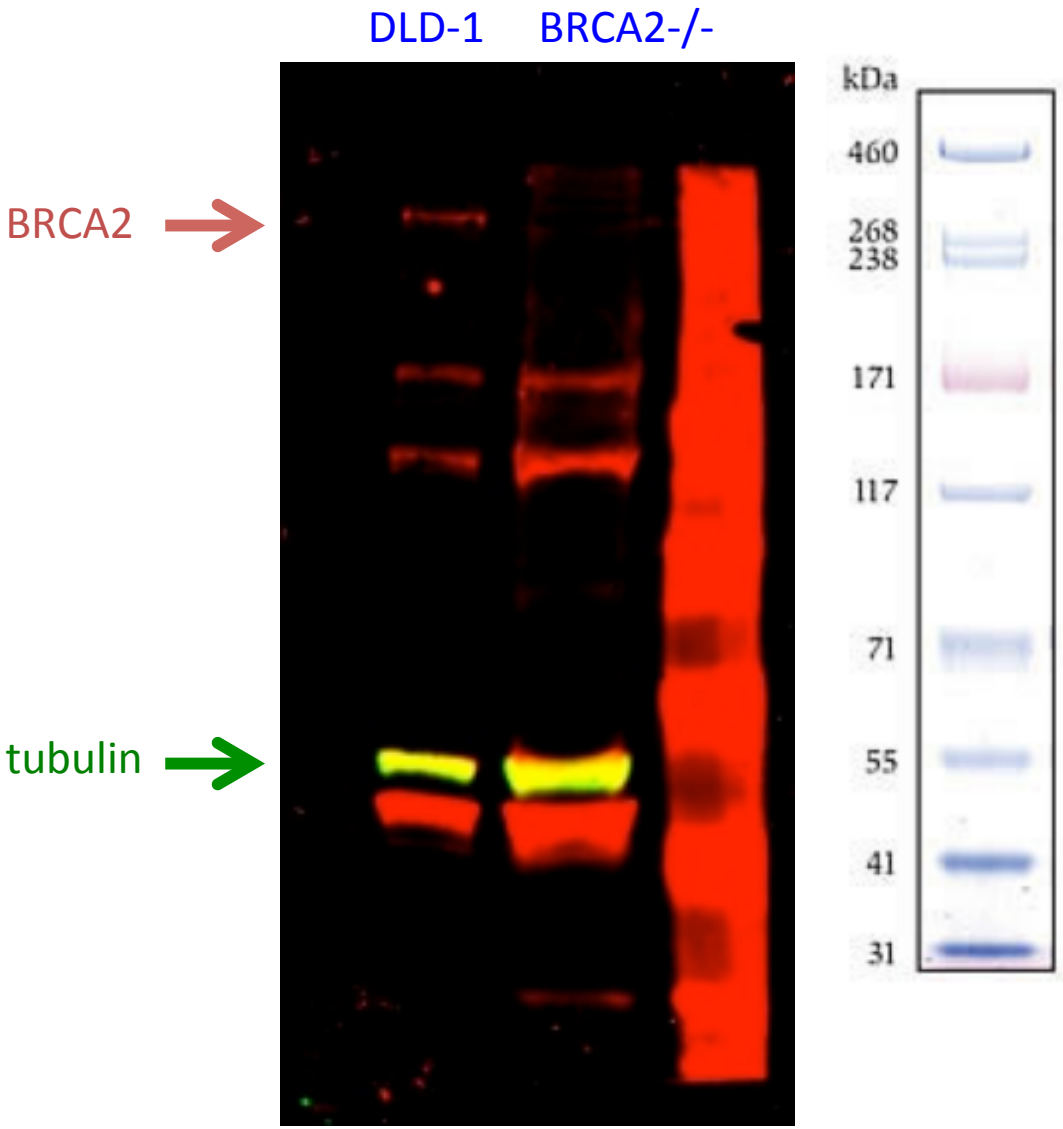
M2D4:Cell viability; quantitative PCR

03/22/2017

1. Quiz
2. Prepare CellTiter Glo assay for cell viability
3. Design qPCR primers
4. Purify cDNA, measure and set up qPCR
5. (Use extra time to revise Mod1 report and ask journal club questions!)

Why are there additional bands on our Western blots?



Monoclonal *vs.* polyclonal antibodies

- Both types created when an antigen is injected into an animal and its immune system responds by producing antibodies specifically targeted against that antigen

- Polyclonal antibodies:
  - mixed pool of immunoglobulin molecules
  - bind to several different epitopes found on a single antigen
  - purified from serum

- Monoclonal antibodies:
  - bind to a single epitope within a target antigen
  - homogeneous cloned immunoglobulin molecules
  - made by fusing antibody-producing B-cells with immortalized B-cells

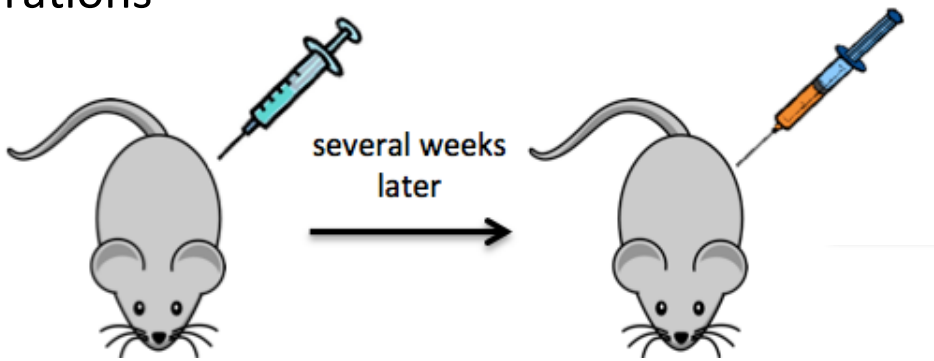
adapted from bio-rad.com

For Western blots, polyclonal antibodies

- Varying specificity to multiple epitopes
- High(er) likelihood to detect the target
- More sensitive: several antibodies per target

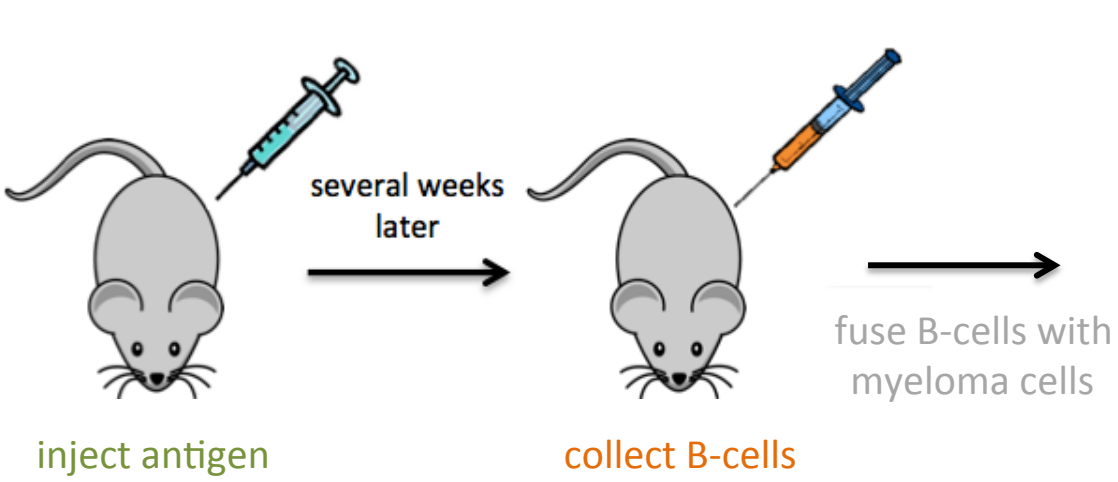
- High(er) background and cross-reactivity (multiple epitopes can be in other proteins)

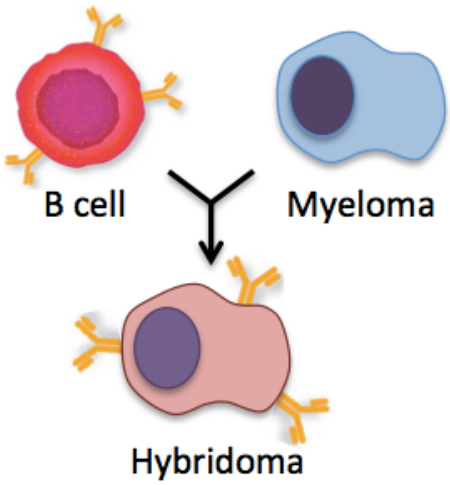
- Less expensive to produce initially, but supply limited to immunized animal
- Greater variability between preparations



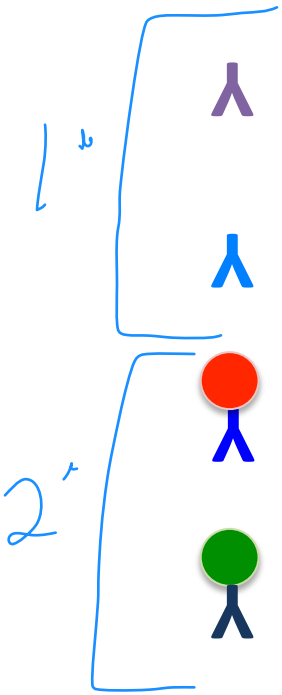
For Western blots, monoclonal antibodies

- Specificity for a single epitope (/region/domain)
- Less sensitive: only one antibody per target
- May cross-react with other proteins that share the recognized domain
- More expensive to produce initially, but available in an unlimited supply





Antibodies used in 20.109



primary anti-BRCA2: rabbit polyclonal

primary anti-tubulin: DM1A, mouse monoclonal

secondary anti-rabbit: donkey polyclonal, *purified**

secondary anti-mouse: goat polyclonal, *purified**

* IgG: immunoglobulin G, most common type of antibody

… for your Discussion

M2D3: you treated cells to examine viability

- etoposide:

creates double-stranded breaks

- compound 401:

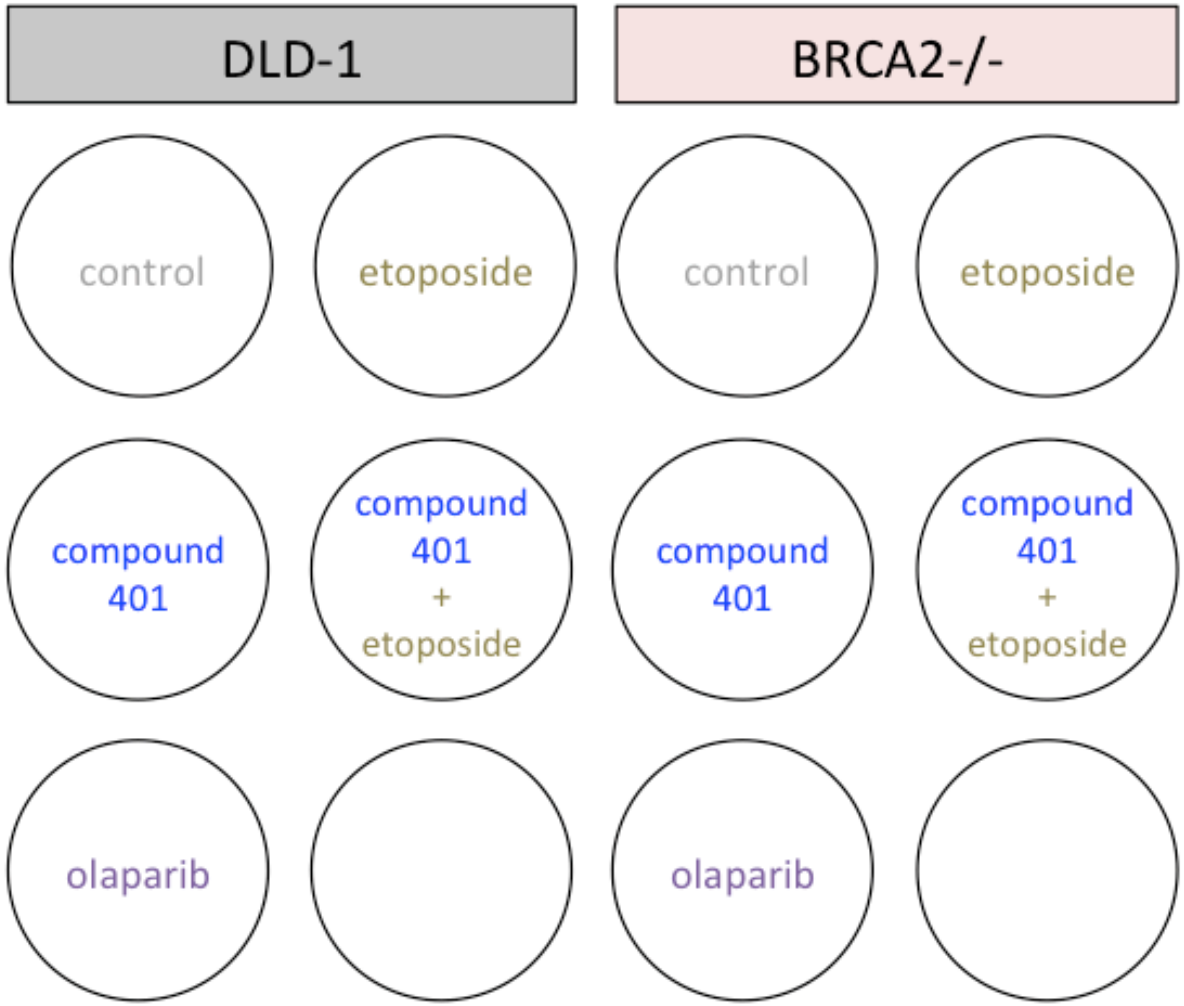
inhibitor of DNA-PK

NHEJ

- olaparib:

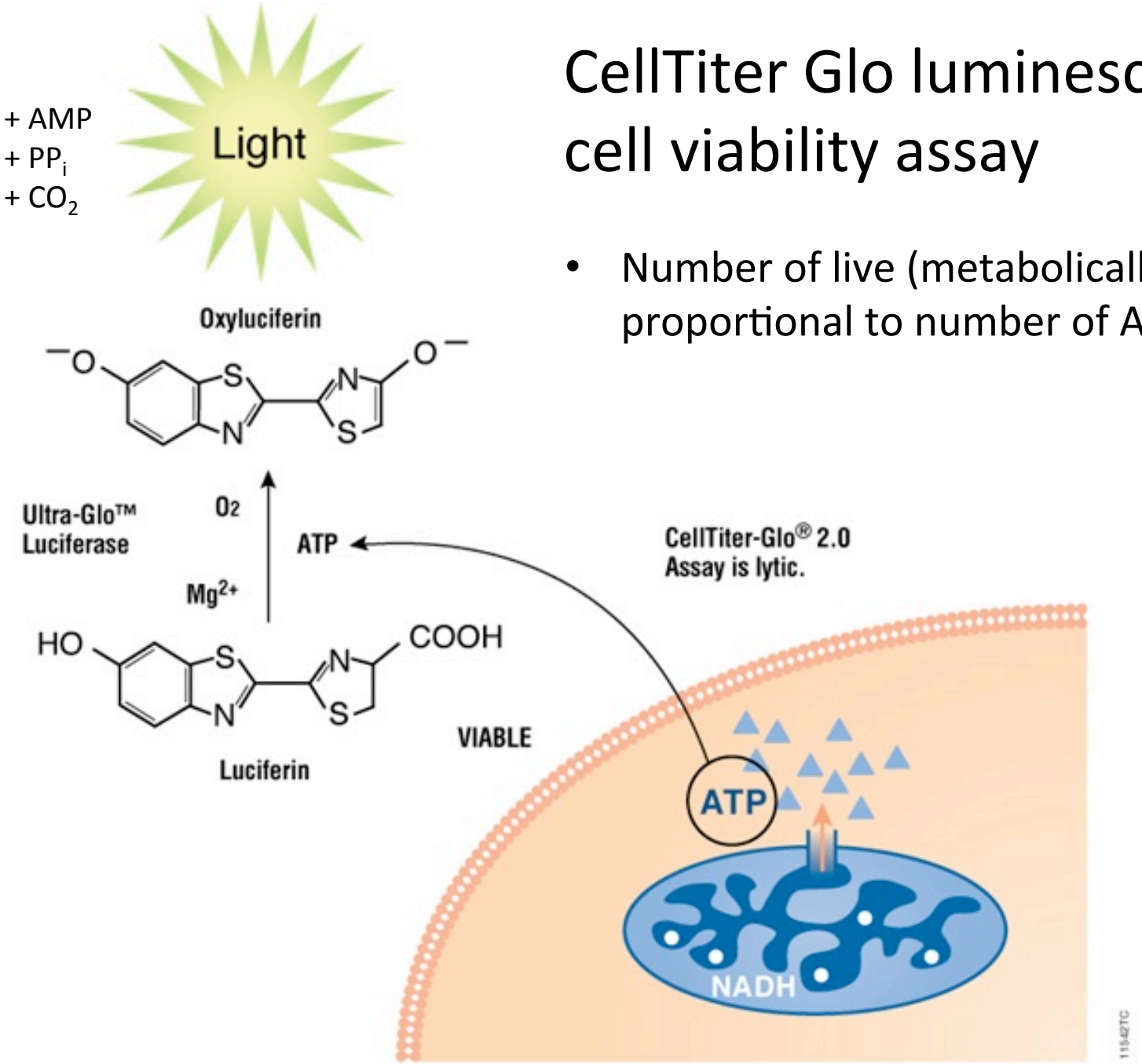
inhibitor of PARP

BER



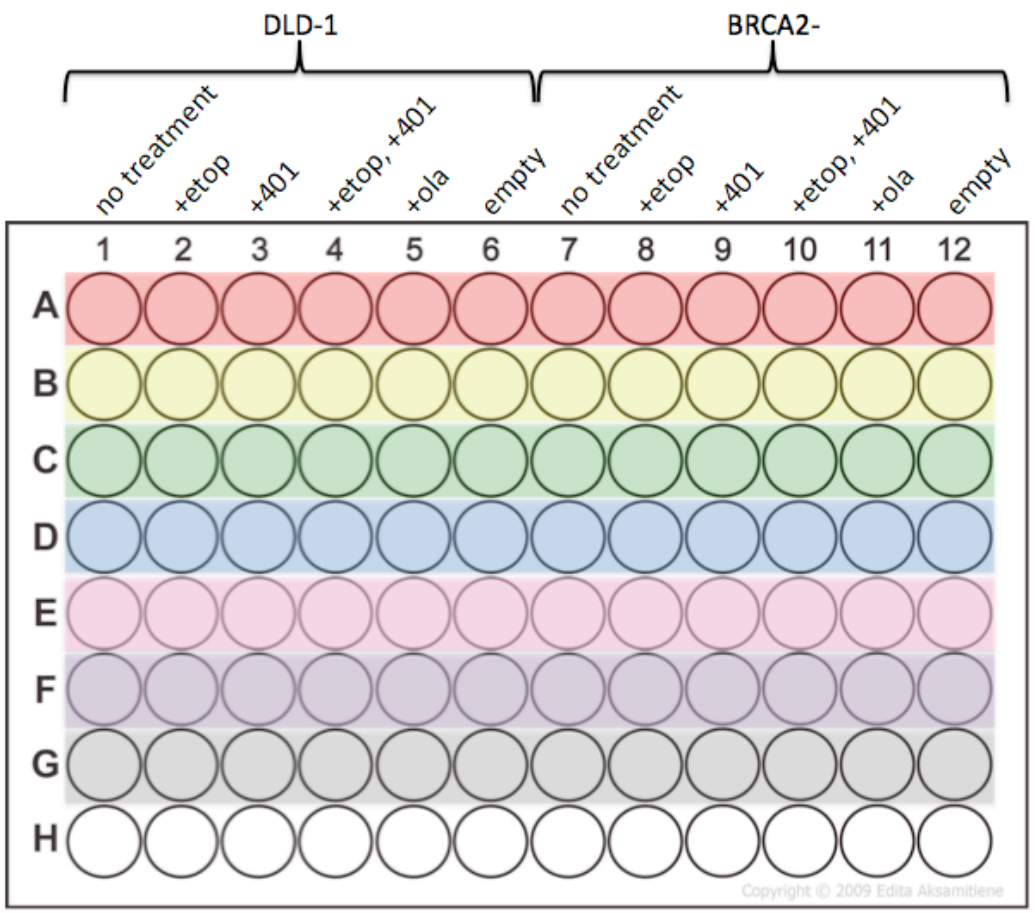
CellTiter Glo luminescent cell viability assay

- Number of live (metabolically active) cells proportional to number of ATP molecules



Practically,

- In 12-well plate,
  - fresh media
  - CellTiter Glo reagent
  - shake & incubate

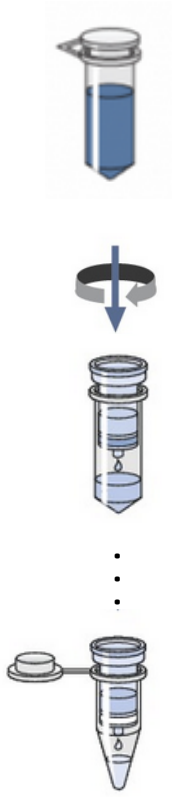


- Transfer to 96-well plate
- Read luminescence with BioMicro Center plate reader

Clean up cDNA : remove all enzyme, buffers + dNTPs

Qiagen QIAquick PCR purification kit

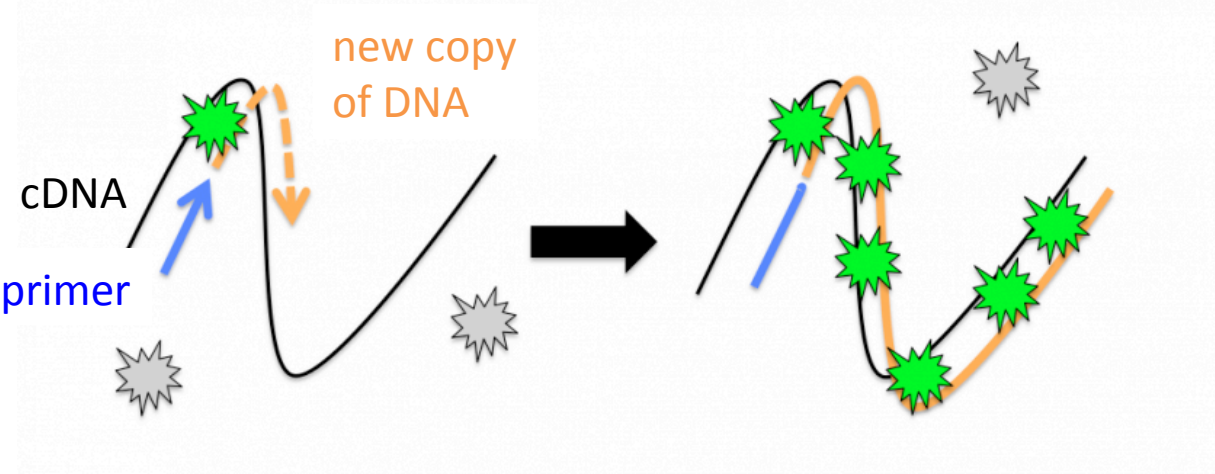
enzyme, dNTPs, buffer and primer (oligo dT)



steps	contents	purpose
prepare	Add 5X Buffer PB to cDNA prep	**high salt (chaotrophic salt) low pH**
bind	silica membrane in column	**DNA binds the silica**
wash	Buffer PE	**ethanol remove contaminants** ** then, get rid of *all* ethanol
elute	water	**high purity DNA**

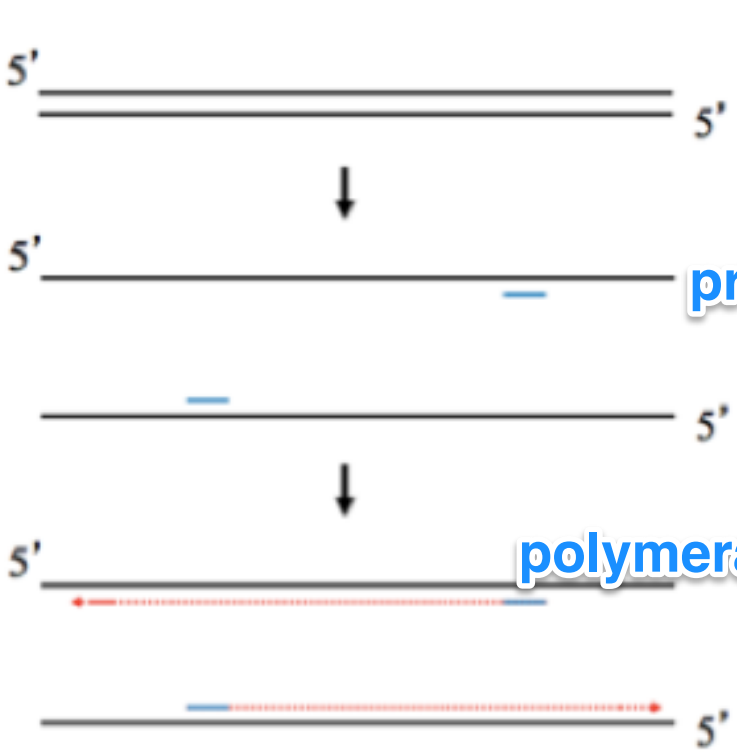
qPCR: quantitative polymerase chain reaction

- Monitor PCR as it occurs
  - using dye (SYBR green) that is fluorescent (green below) when bound to double stranded DNA; little or no fluorescence when not bound to dsDNA (grey below)
  - signal proportional to initial amount of cDNA (= original RNA = **gene expression**)



qPCR ingredients and cycling conditions

PCR ingredients
SYBR green
cDNA template
buffer and water
sequence-specific primers
DNA polymerase
dNTPs



melt dsDNA
95 °C
(15 s)

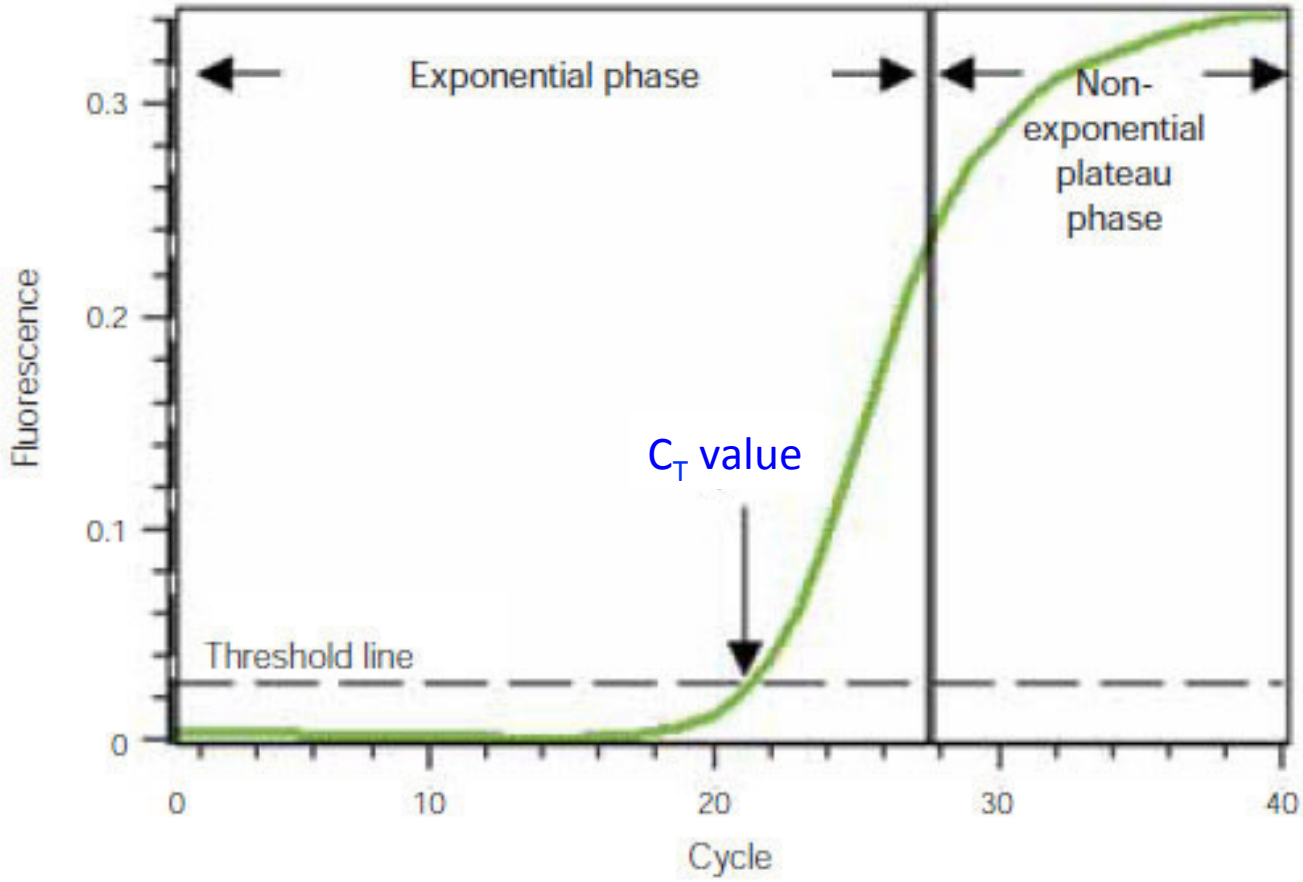
primers anneal
60 °C
(30 s)

polymerase extension
72 °C
(30 s)

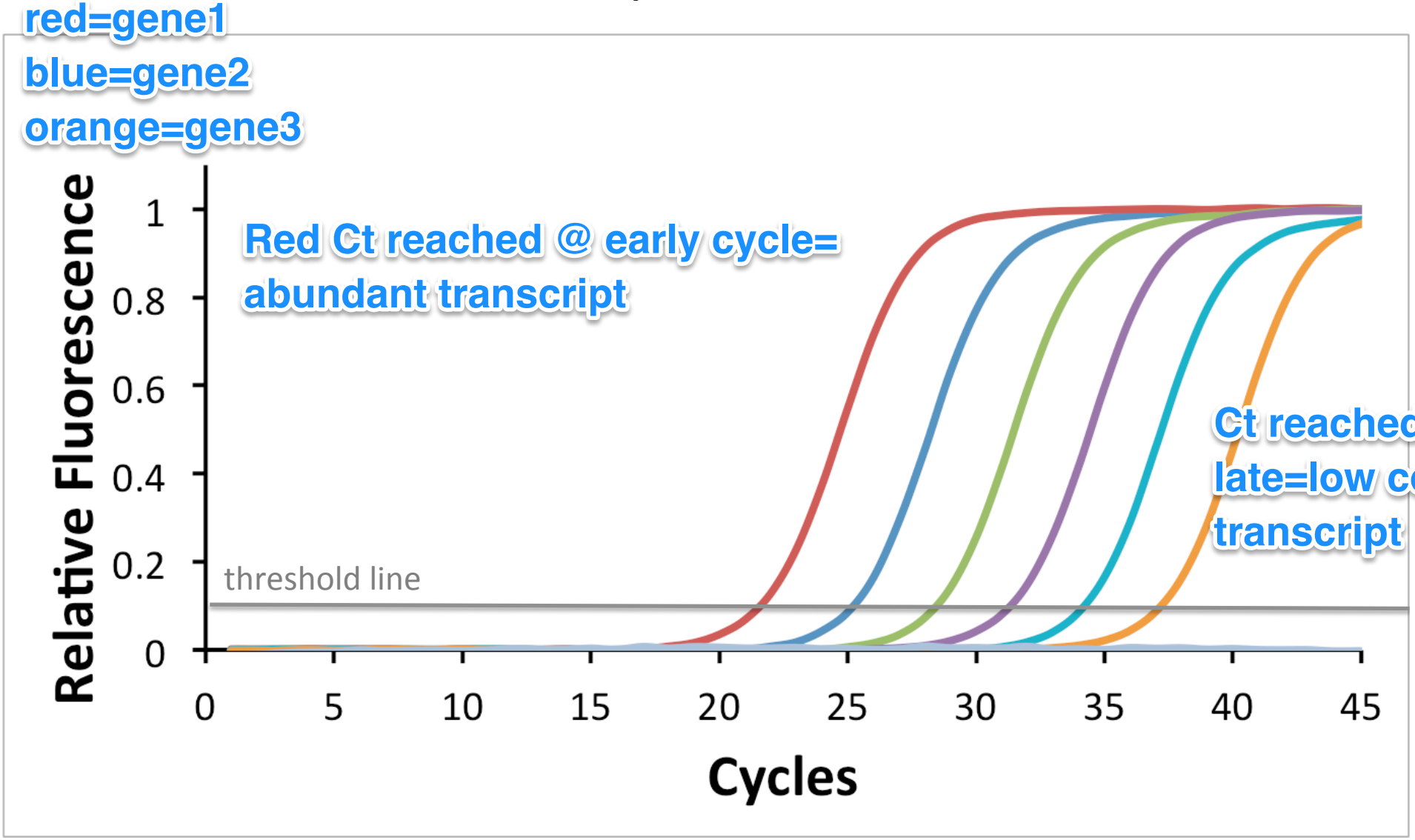
40 cycles

qPCR data output

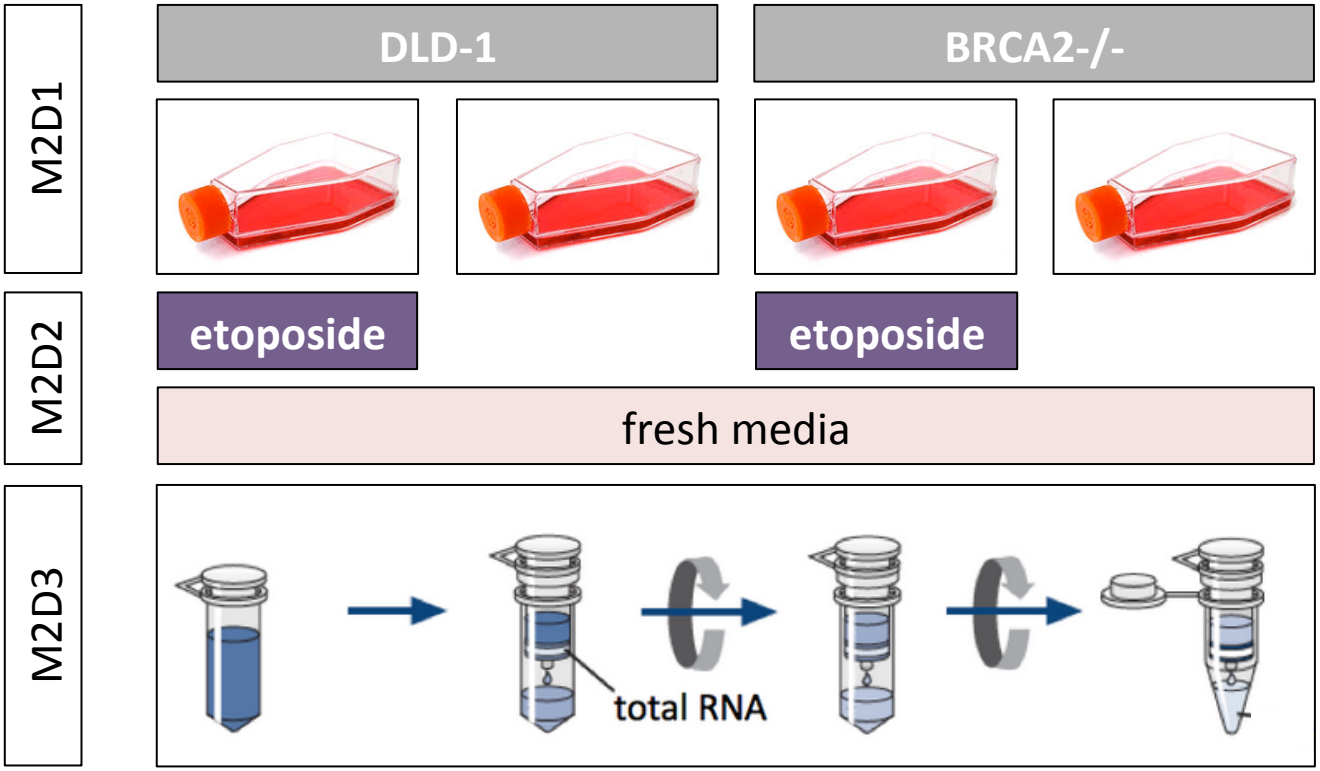
- Plotted as Fluorescence *vs*. cycle number
- C_T threshold cycle
  - fluorescent signal significantly above the background fluorescence
  - relative measure of the initial number of copies of template cDNA



C_T value related to amount of template present at the start of the amplification reaction



How does cancer chemotherapy affect gene expression?



and cDNA

quantify levels of GAPDH
p21

Why look at this subset of transcripts?

- p21 (also CDKN1A)
  - regulator of cell cycle progression at G1; arrest in G1 in response to stress
  - tightly controlled by tumor suppressor p53

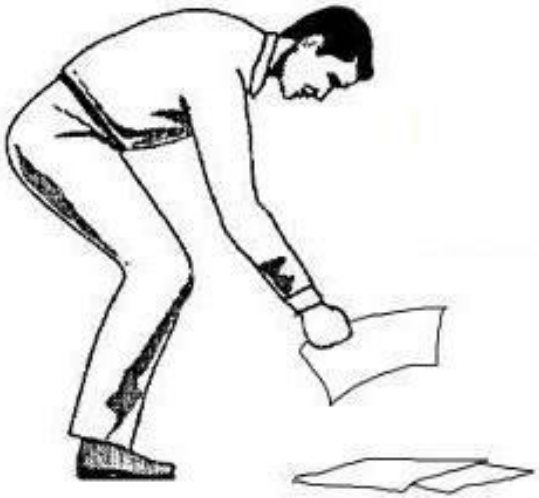
- GAPDH (glyceraldehyde 3-phosphate dehydrogenase)
  - catalyzes glycolysis
  - housekeeping gene: stably, constitutively, highly expressed in cells

- BRCA2 exon 11

Today in lab:

1. Prepare CellTiter Glo assay
2. Go through qPCR primer design exercise
3. Clean cDNA prep with QIAquick kit
4. Prepare qPCR mastermix for each reaction
5. Use the extra lab time well

- No homework due Friday
- Journal club presentations in 16-336
  - JC1 presenters upload your slides to Stellar by 1pm Friday



- M1 Data Summary revision due Monday, March 27
- M1 blog post due Monday, April 3
- M2D6 homework: M2D1-M2D4 Methods