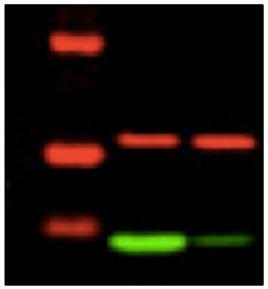
M2D3:
Finish Western blot;
Extract RNA for qPCR assay;
Treat cells for viability assay.

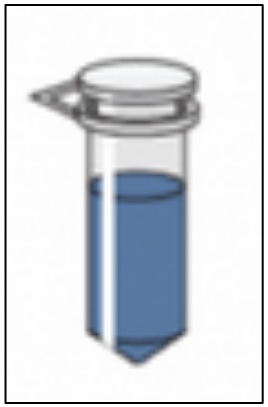
03/16/2017

In lab today



Student # 1 takes the lead

1. Finish Western blot analysis of cell lines



Student # 2 takes the lead

2. Prepare qPCR samples
   - extract RNA from DLD-1 BRCA2-/- +/- etoposide
   - synthesize cDNA

Student # 1 takes the lead

3. Treat cells for viability assay
   - Compound 401 (+/- etoposide)
   - olaparib

Key assignments of M2

- Journal club presentation video-recorded
  - 15%
  - individual
  - in class at 1pm on March 23 or April 11

- Research article
  - 20%
  - individual
  - due 10pm on April 22
  - no draft/revision this time around

From Prof. Samson's lecture 03/09/17:

What experimental question will you ask in Module 2?

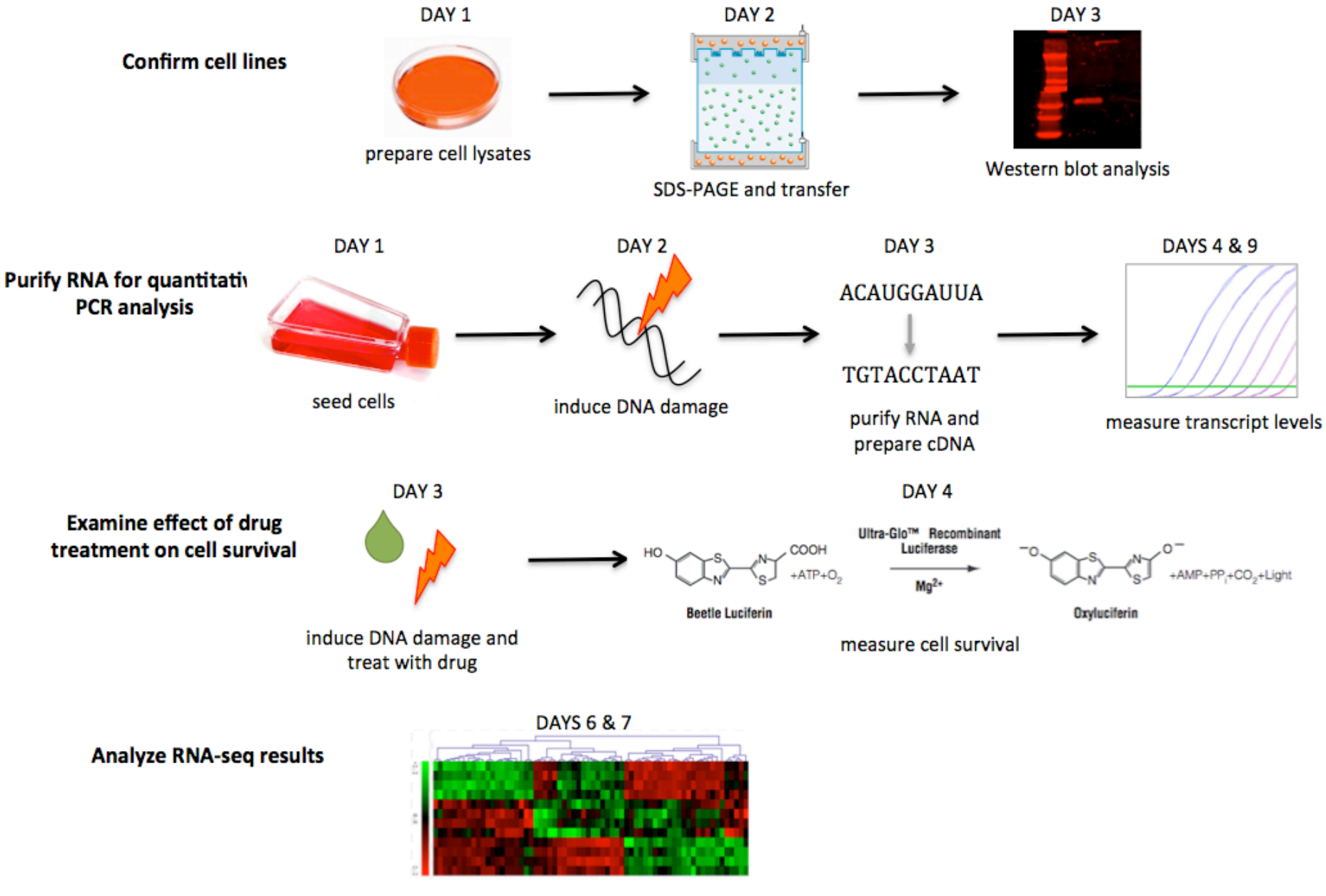
How does DNA repair affect the ability of cancer chemotherapy drugs to kill cancer cells?

How does cancer chemotherapy affect gene expression?

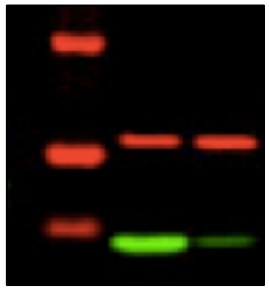
This raises the following questions

- How does DNA get damaged?
- What is DNA repair?
- Why does DNA repair exist?

M2: Experimental overview



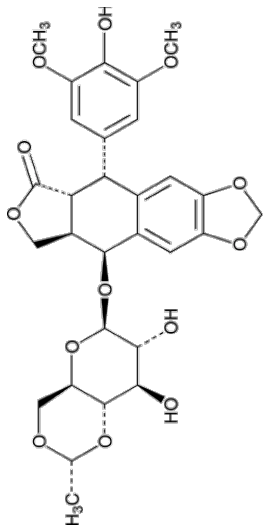
In lab on snow day (M2D2)



1. Verify cell lines by Western blot protein analysis:

"immunofluorescence on PAGE-separated proteins"

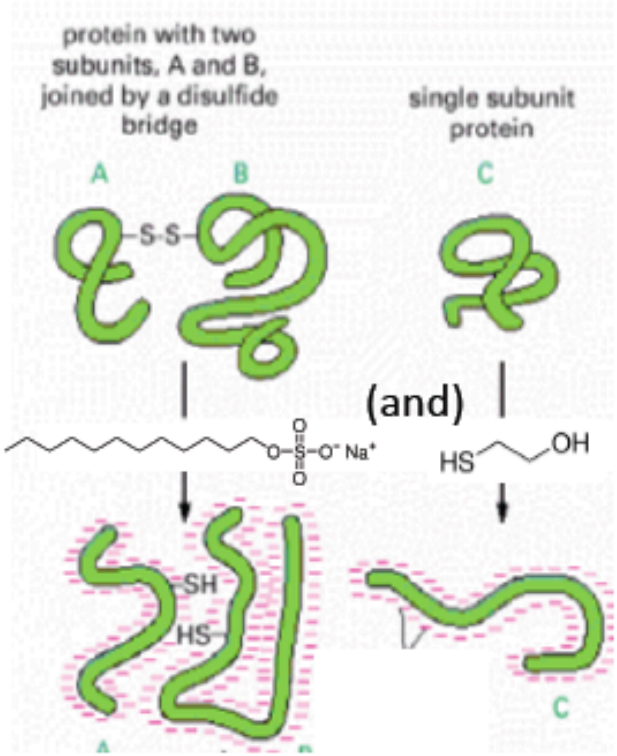
   - Lyse DLD-1 and BRCA2-/- cells
   - Measure protein concentration
   - Separate proteins by SDS-PAGE
   - Transfer proteins onto nitrocellulose membrane
   - Label BRCA2 with primary+secondary antibodies



2. Treat cells with cancer drug etoposide

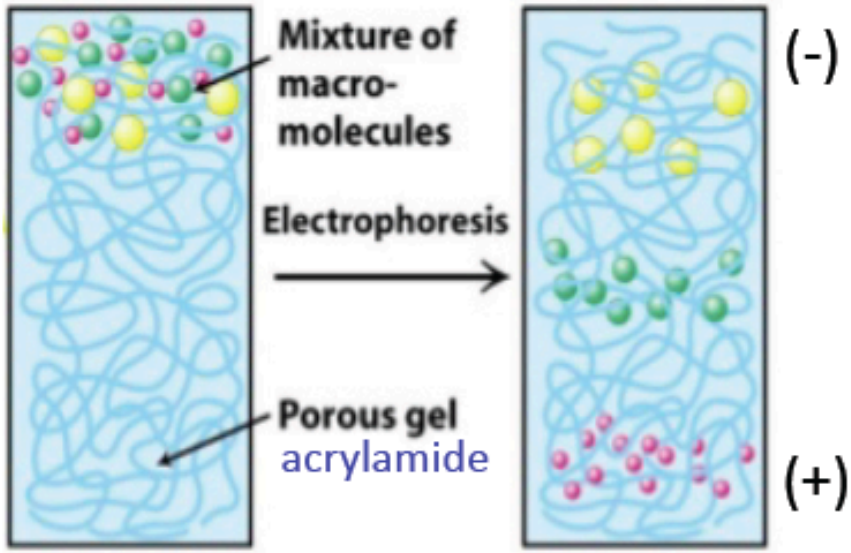
Review: SDS-PAGE

- What gives proteins uniform charge and linear structure?



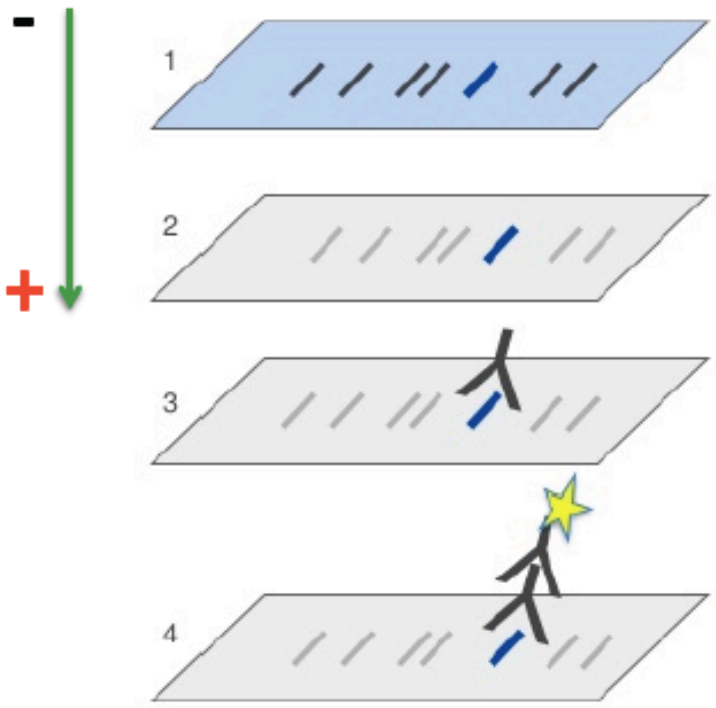
Laemmli buffer's
SDS + beta-mercaptoethanol

- How are proteins separated?



by size (now proportional to charge)

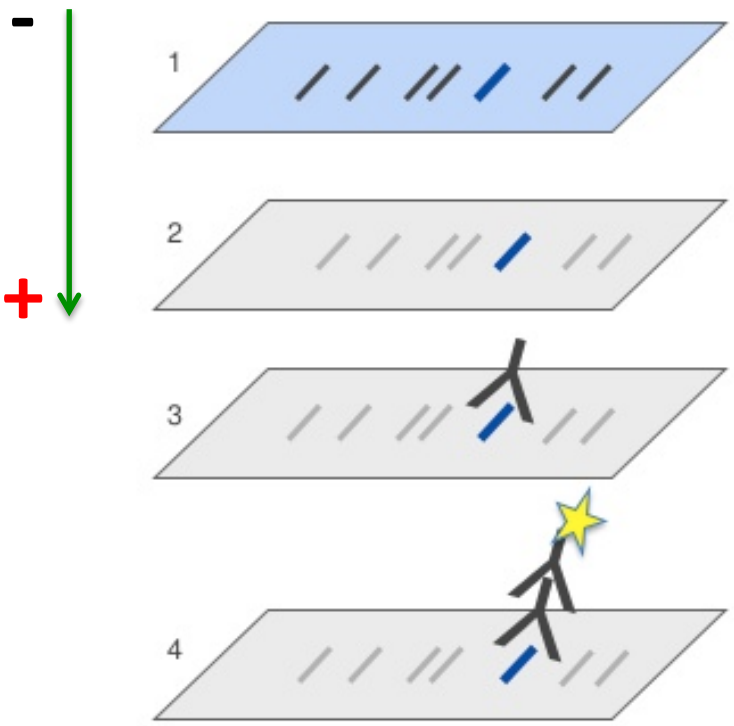
Western blot workflow on snow day



1. Protein separation by SDS-PAGE
   - HiMark stained ladder bands 31-460 kDa
2. Protein transfer to nitrocellulose membrane
   - high affinity for proteins
   - immobilizes proteins
3. (Blocking and) probing with primary antibodies specific to
   - BRCA2 (380 kDa)
   - tubulin
4. Probing with labeled secondary antibodies specific to primary antibodies
5. Image fluorescence signal

from GE Healthcare

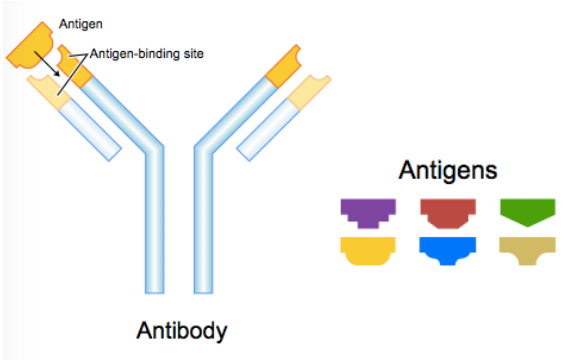
Western blot workflow: today



1. Protein separation by SDS-PAGE
2. Protein transfer to nitrocellulose membrane
3. Block membrane address non-specific binding
4. Probe with primary antibodies specific to
   - BRCA2 24 hours ago
   - tubulin
5. Wash with TBS-T Tris-buffered saline
   - Tween, a mild detergent
   - to get rid of unbound primary antibody
   - and to detach weak interactions
6. Probe with labeled secondary antibodies specific to primary antibodies
7. Wash
8. Image *LI-COR* fluorescence signal

from GE Healthcare

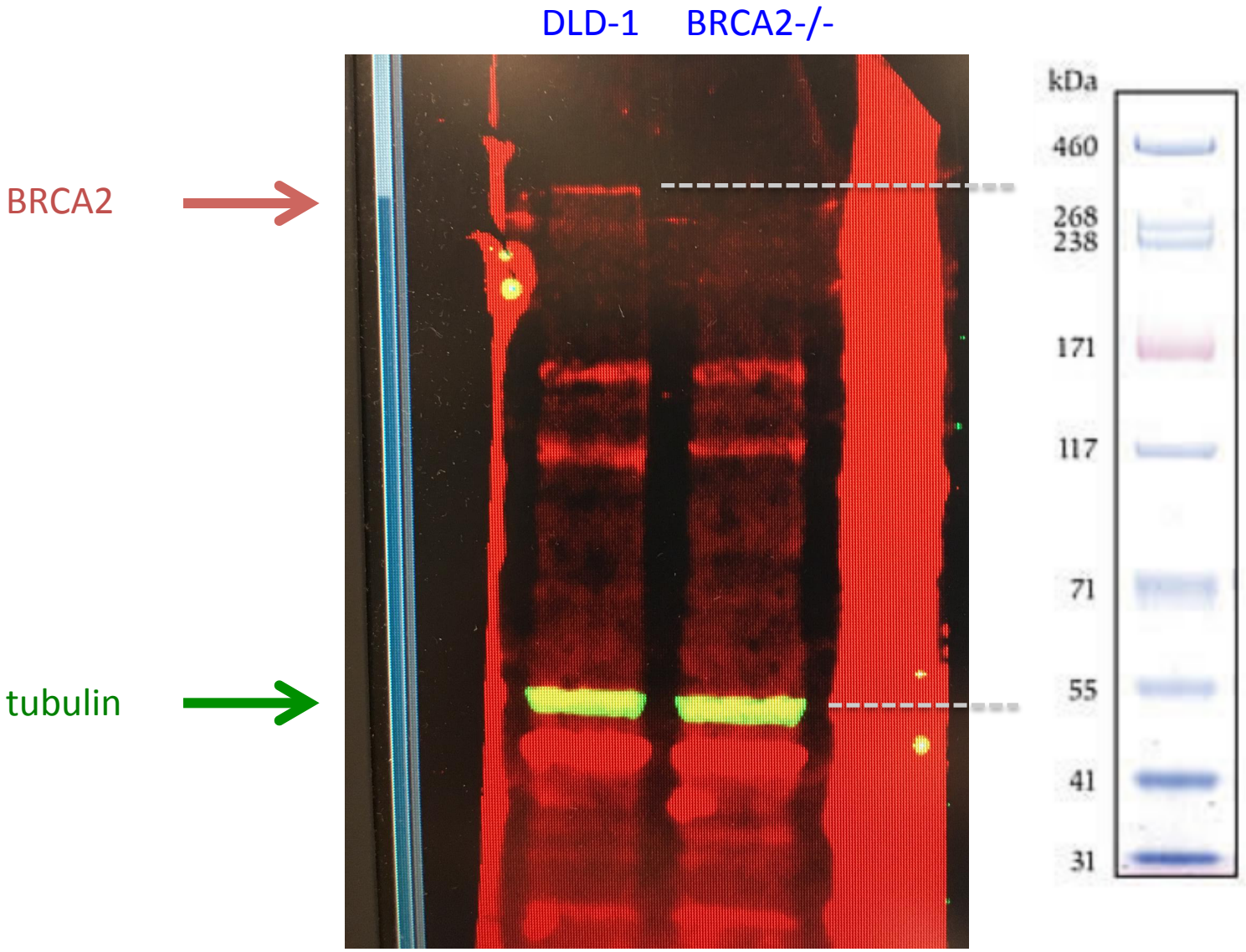
Suite of antibodies for *LI-COR* Western blot



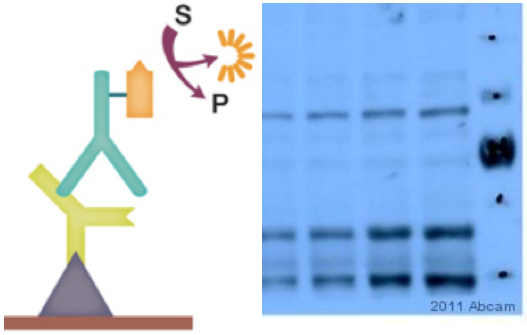
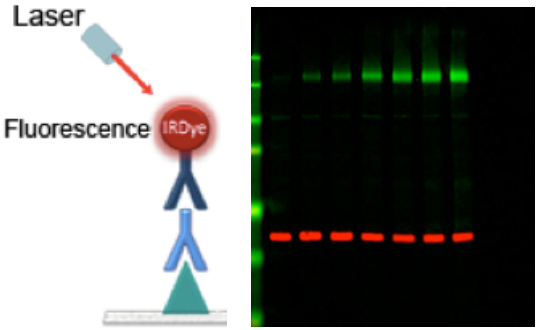
antigens

protein of interest	tubulin	BRCA2
primary antibody	mouse anti-human anti-tubulin	rabbit anti-human anti-BRCA2
secondary antibody	goat anti-mouse	donkey anti-rabbit
fluorescent dye IR wavelength	800 nm	680 nm
pseudo-color	green	red
molecular weight	~ 50 kDa	~ 380 kDa

Verify BRCA2 is missing by *LI-COR* Western blot



Western blot detection / visualization

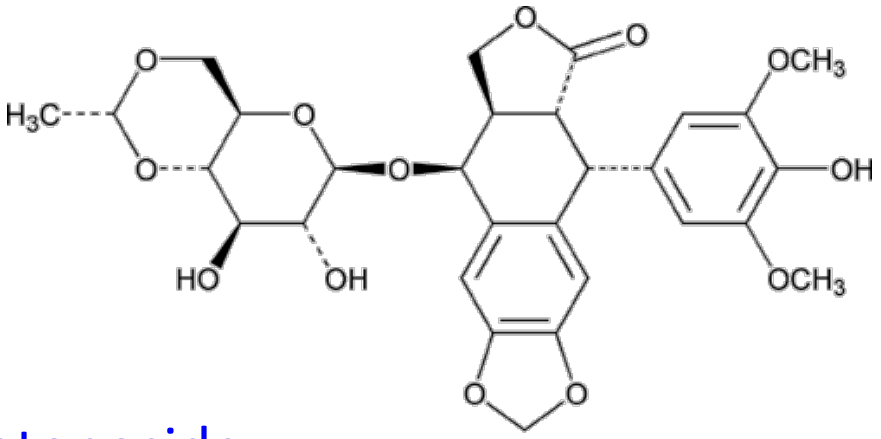
Colorimetric	Chemi-luminescent	Fluorescent (Li-COR)
		
Upon incubation with a substrate that reacts with reporter (*e.g.* peroxidase), dye rendered insoluble and colored precipitates on membrane.	Incubation substrate luminesces when exposed to reporter on secondary antibody.	The fluorescently labeled probe is excited by light and the fluorescence emission is detected by a photosensor such as a CCD camera.
Pro: inexpensive, easy, no equipment required	**Pro**: sensitive, fast, film developer is common	**Pro**: sensitive, stable, able to multiplex
Con: medium sensitivity	**Con**: requires trial and error, time-dependent snapshot	**Con**: expensive

Homework due M2D4: use Western blot

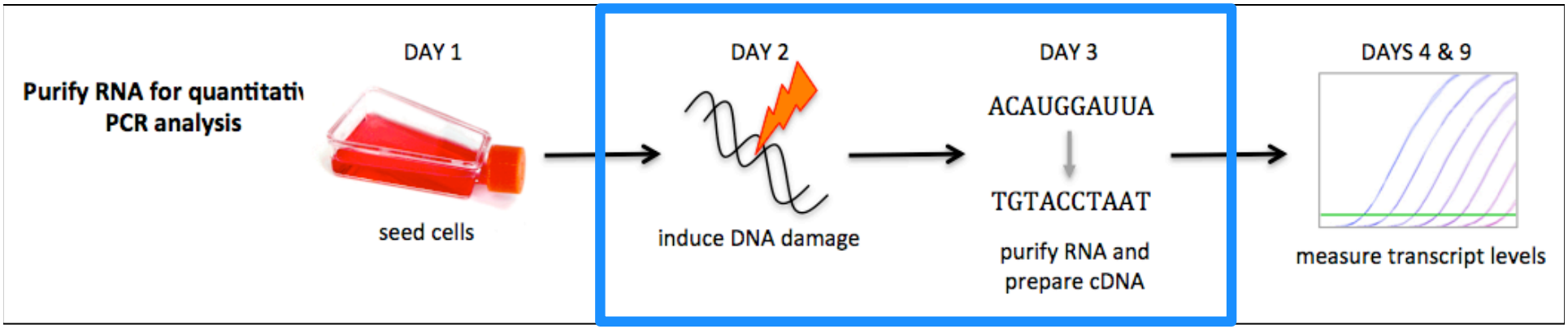
- Figure (with title and caption)
- Results (no more bullet points, paragraph form)
  - one title (but several paragraphs for several topics)
  - introductory topic sentence
  - state findings
  - conclude / transition to next result

- (Discussion / interpretation will be separate in M2 research article)

Also on snow day

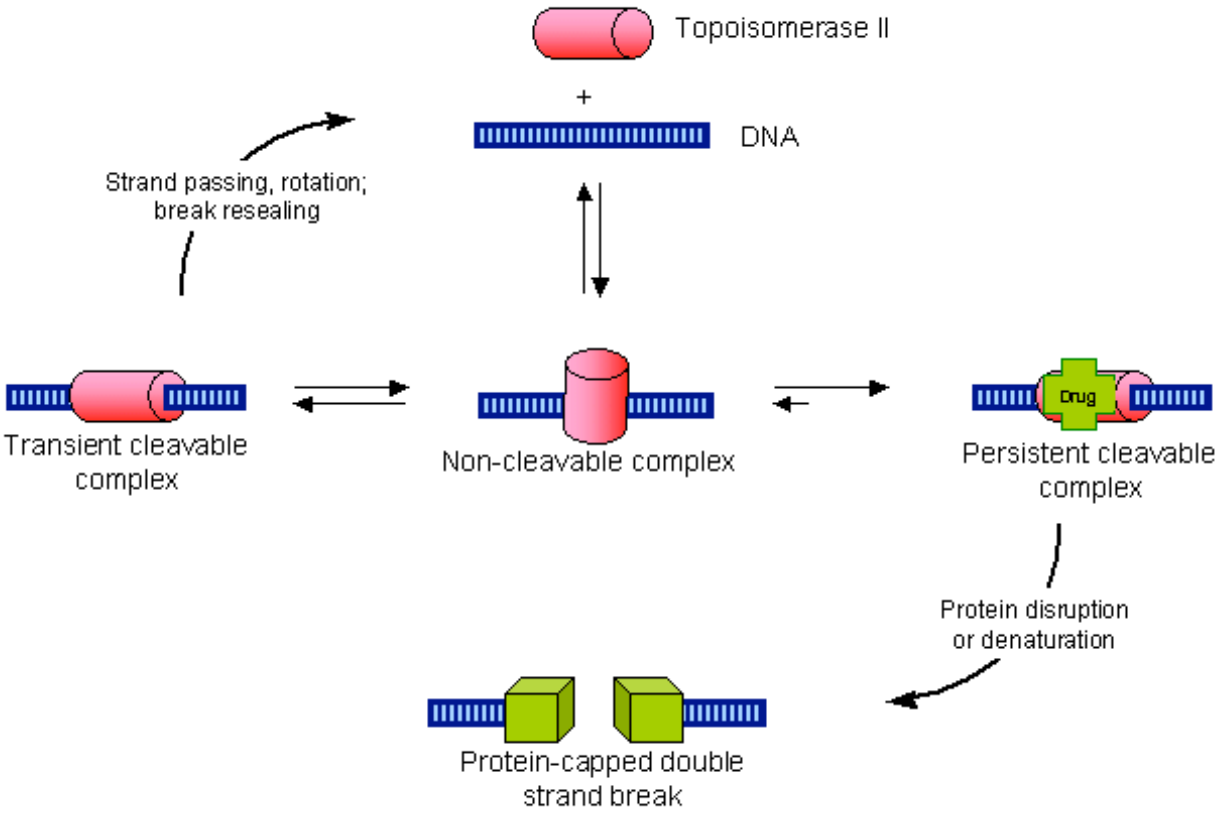


2. Treat cells with cancer drug etoposide



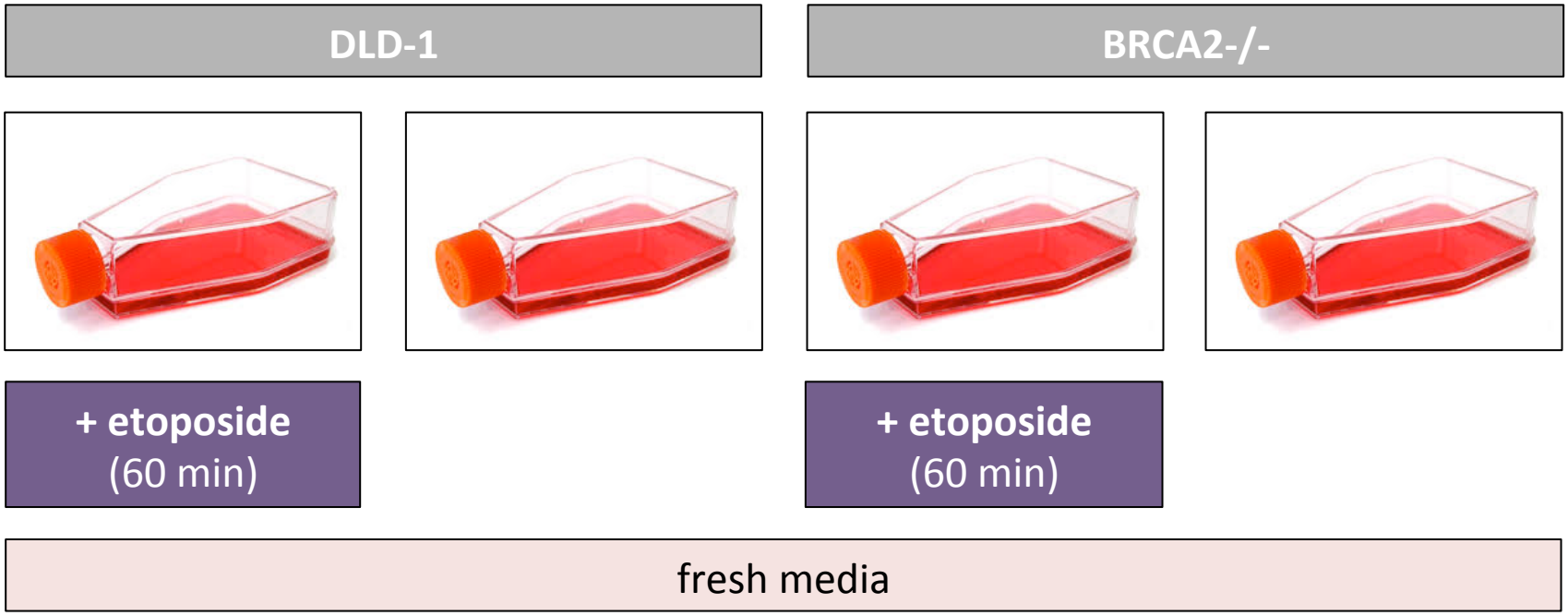
Etoposide's mechanism of action

- forms a ternary complex with DNA and the topoisomerase II enzyme (which aids in DNA unwinding), to remove supercoils of DNA
- prevents re-ligation of the DNA strands,
- so causes DNA strands to break,
- and (cancer) cells to undergo apoptosis.



Treat cells with etoposide

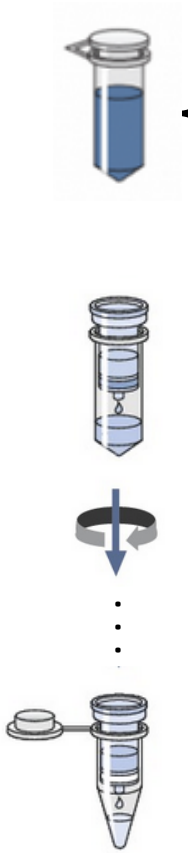
Thank you, Noreen!



M2D3: extract RNA today

Has cancer drug affected transcriptional profiles / gene expression?

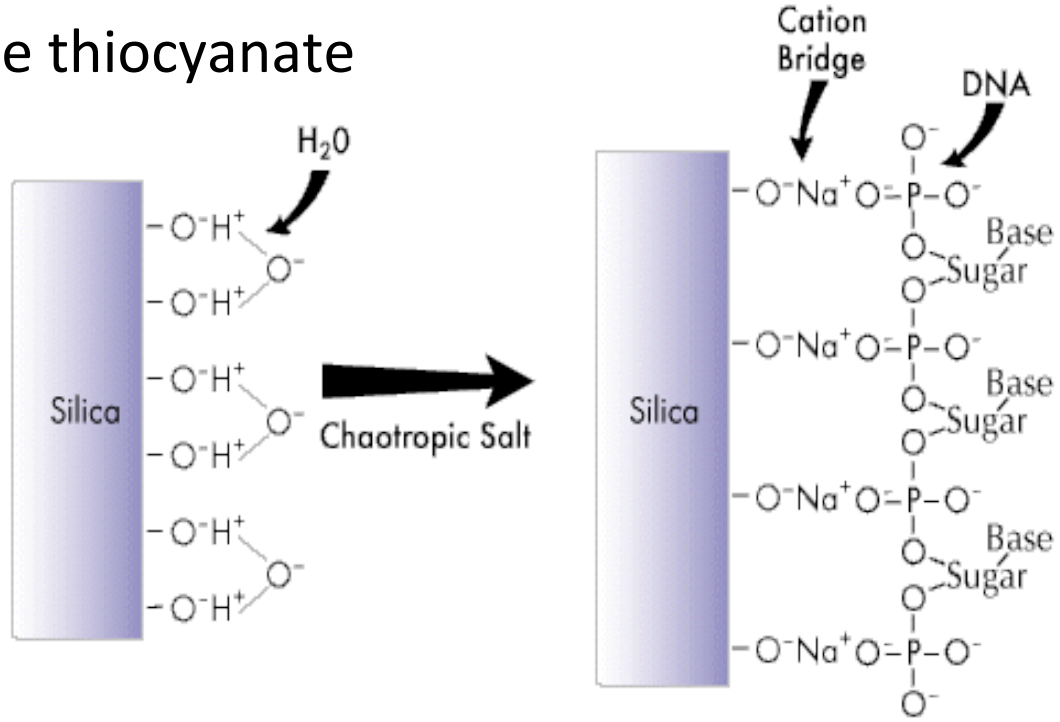
Isolate RNA: QIAshredder + Rneasy kit



steps	contents	purpose
lyse	RLT (with highly denaturing guanidine-thiocyanate salt) detergents + QIAshredder	inactivate RNase, disrupt membranes, helps bind column homogenize (shear high-MW genomic DNA)
prepare	ethanol	promote efficient binding
bind	silica membrane in column, retain mRNA (> 200 nucleotides)	
wash	RW1 RPE	get rid of contaminants ** then, get rid of *all* ethanol
elute	water, RNase-free	high-purity RNA

Chaotropic salts help DNA/RNA bind to column

e.g. guanidine thiocyanate



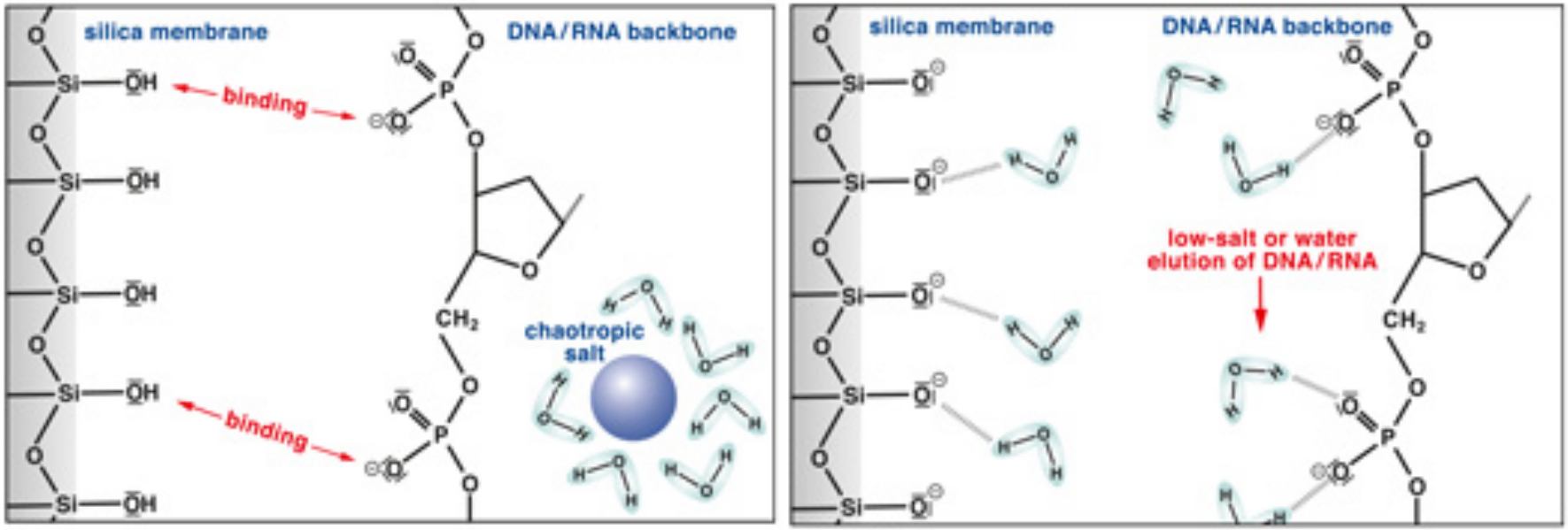
- Washes with RW1 and RPE remove residual contaminants
  - RW1 contains a guanidine salt, as well as ethanol, and is used as a stringent washing buffer that efficiently removes biomolecules such as carbohydrates, proteins, fatty acids, etc, that are non-specifically bound to the silica membrane
  - RPE contains ethanol and is a mild washing buffer

from Qiagen

Water is used to elute nucleic acids

or low ionic-strength solvent

- Water competes RNA off of column



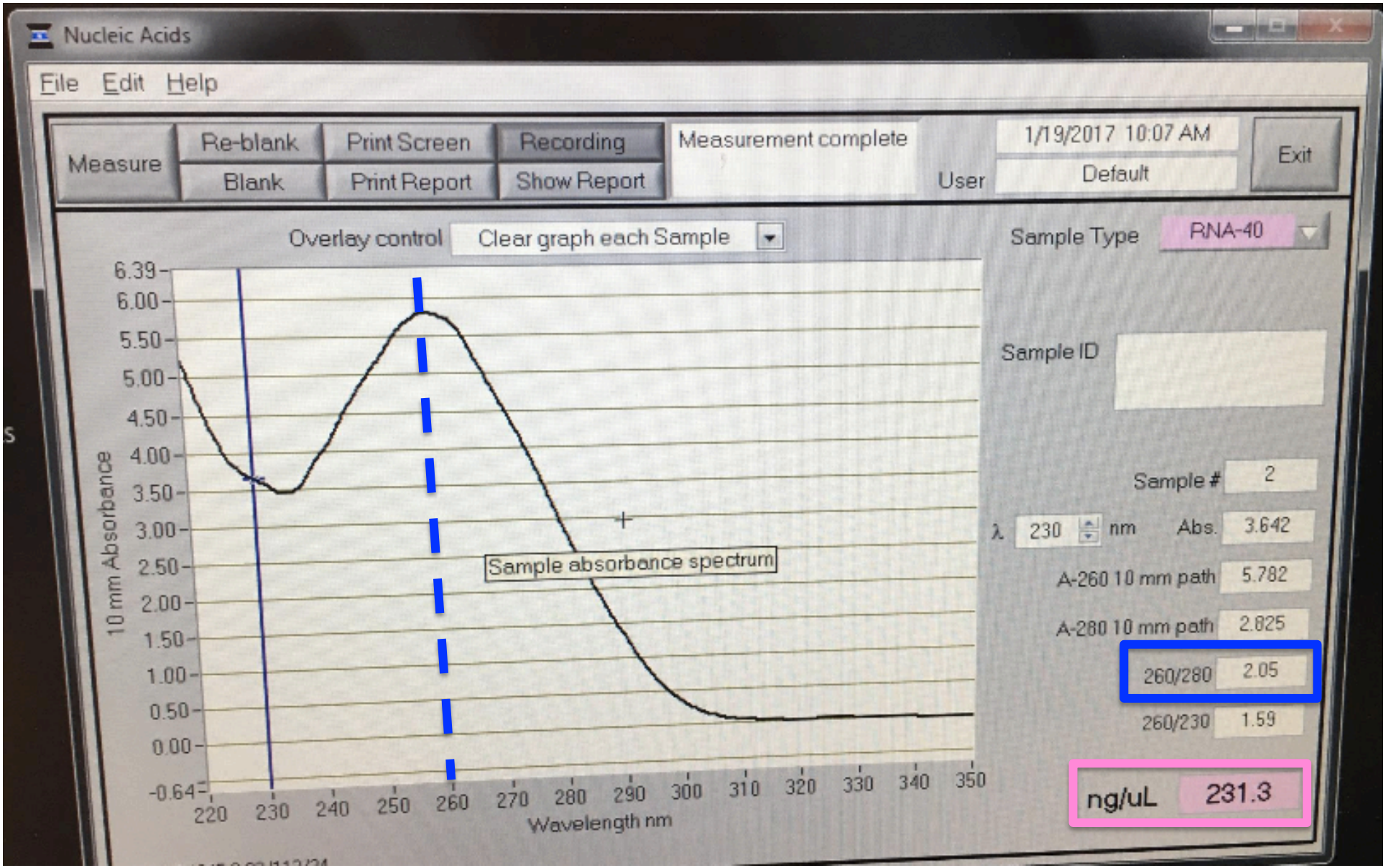
before elution

at elution

RNA concentration from NanoDrop spectrophotometer

- A_{260}/A_{280}
  - nucleic acids absorb at 260 nm
  - proteins absorb at 280 nm
  - ratio ~ 1.8 "pure" DNA
  - ratio ~ 2.0 "pure" RNA
  - note: A_{230} from contaminants (phenol, guanidine, carbohydrates,..)

RNA concentration from NanoDrop

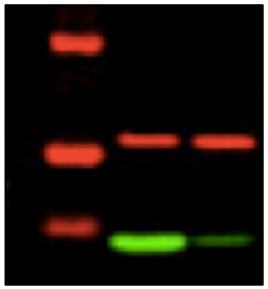


Synthesize cDNA from purified RNA

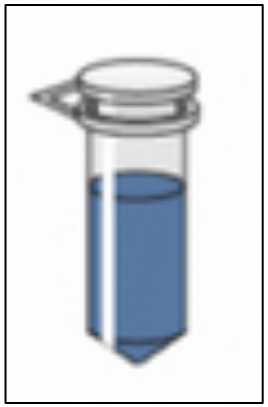
- cDNA: complementary DNA
- RT-PCR: reverse transcription polymerase chain reaction

steps	conditions	ingredients added
denature	65°C 5 min on ice 1 min	1 µg RNA + primers + dNTPs
anneal	oligo $(dT)_{20}$	primer: oligo $(dT)_{20}$
synthesize	50°C 50 min	SuperScript III RT, RNaseOUT, $MgCl_2$, DTT, buffer
terminate	85°C 5 min	
remove RNA	37°C 20 min	RNase H
PCR amplify	see M2D4	

In lab today



1. Finish Western blot analysis of cell lines



2. Prepare qPCR samples
   - extract RNA from DLD-1 BRCA2-/- +/- etoposide
   - synthesize cDNA

3. Treat cells for viability assay
   - Compound 401
   - olaparib

Treat cells to examine viability

- etoposide:

chemo-therapeutic drug

- compound 401:

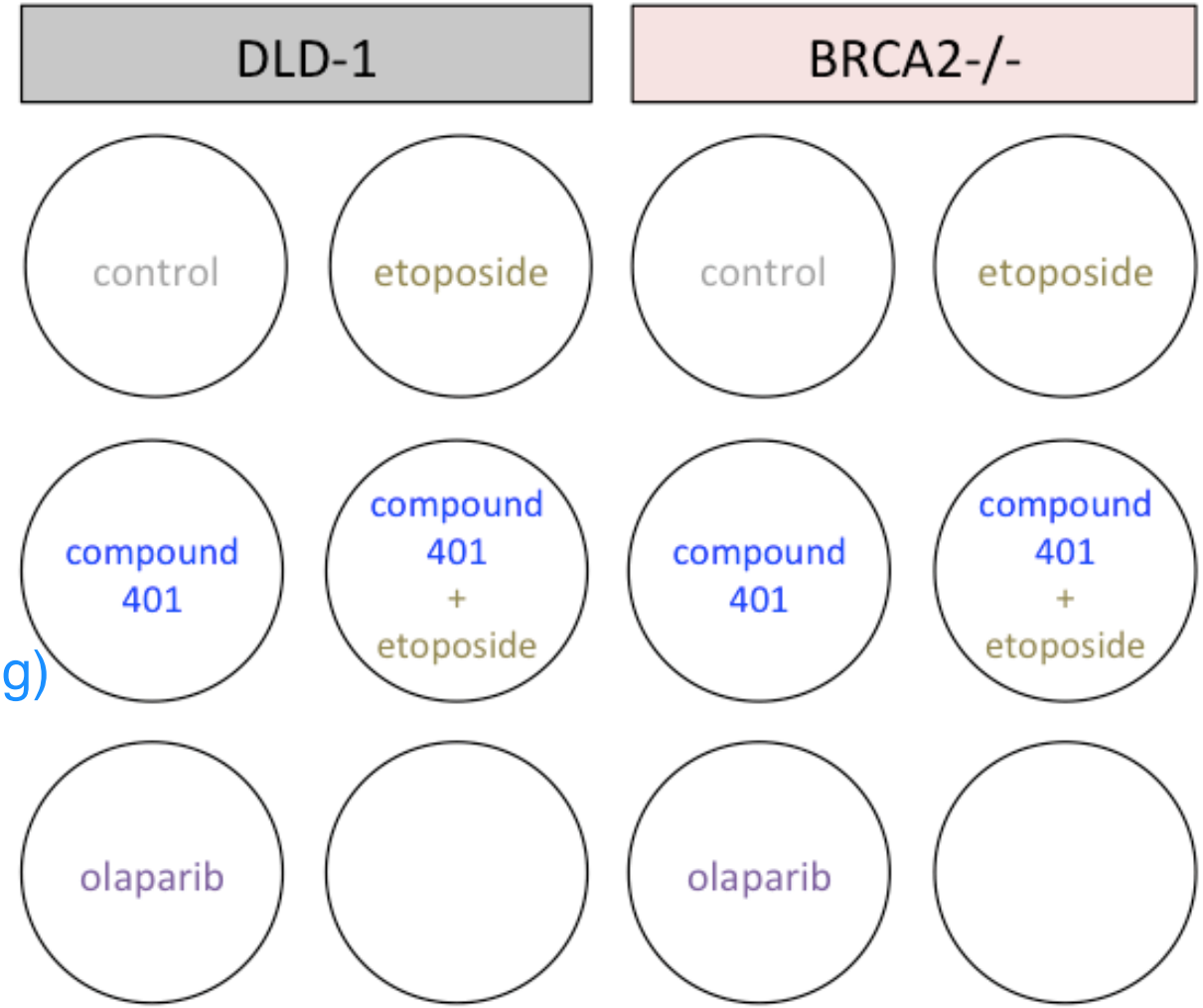
inhibitor of DNA-PK

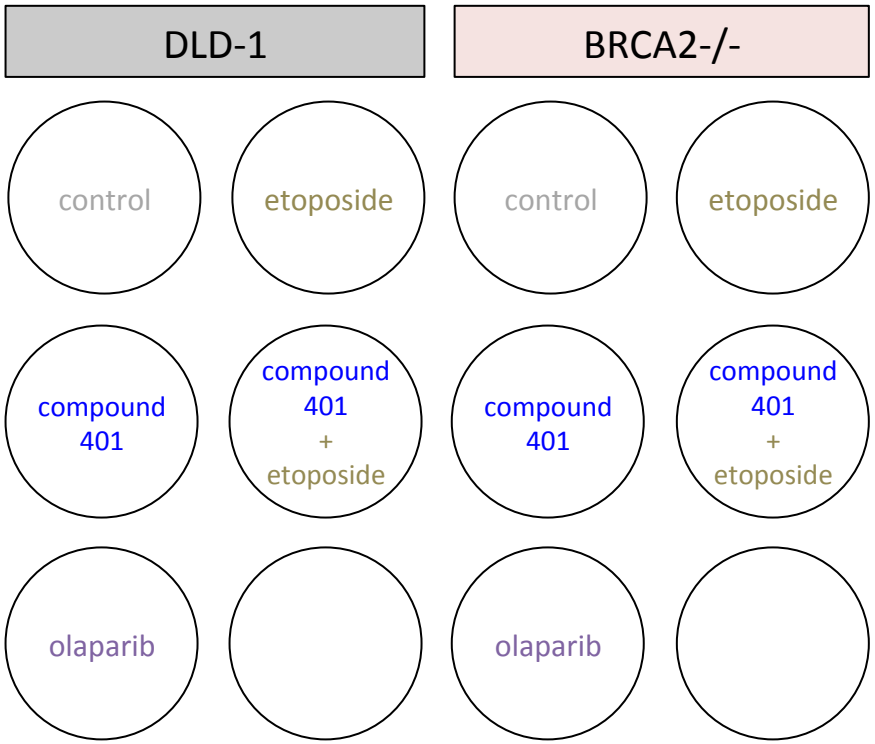
inhibitor of NHEJ
(non-homologous end joining)

- olaparib:

PARP inhibitor (poly ADP ribose polymerase)

inhibitor of BER
(base excision repair)




DLD-1
BRCA2-/-
control
etoposide
control
etoposide
compound 401
compound 401 + etoposide
compound 401
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olaparib
olaparib