M2D8: Cell viability, quantitative PCR, identification of regulatory motifs

1. Treat cells with DNA damaging agents and inhibitors for cell viability
2. Analyze qPCR results
3. R: Identify regulatory motifs in RNA-seq data

Extra (+ usual) Office Hours Next Week

- Tuesday April 17th 56-322 (lab):
  - 10:30am-1:30pm (Leslie)
  - 2:00pm-5:00pm (Noreen + Josephine's regular hour)
- Wednesday April 18th 56-322 (lab):
  - 10:00am-1:00pm (Josephine)
  - 2:00pm-4:00pm (Noreen) 4-5pm (Leslie)
- Thursday April 19th (56-341c), 10-11am (Josephine)
- Friday April 20th (56-341c), 4-5pm (Leslie)

Mod2 Research Report (20% of final grade)

Due Saturday 4/21 at 10pm

- Title, Abstract
- Introduction
- Methods
- Results (Figures and captions)
- Discussion
- References

Last week of Mod2!

Purify RNA for quantitative PCR analysis

DAY 1

seed cells

DAY 2

induce DNA damage

DAY 3

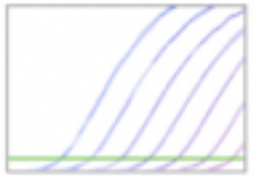
ACAUGGAUUA

↓

TGTACCTAAT

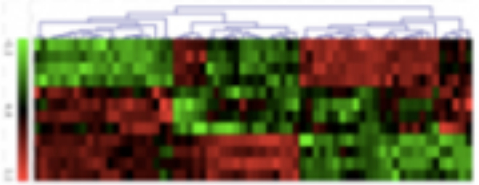
purify RNA and prepare cDNA

DAY 5



measure transcript levels

Analyze RNA-seq results



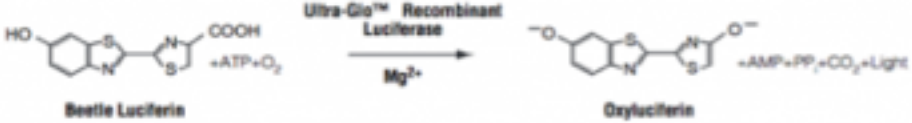
DAY 4: Evaluate altered gene expression
DAY 5: Investigate public databases
DAY 8: Identify regulatory motifs

Examine effect of drug treatment on cell survival

DAY 8

induce DNA damage and treat with drug

DAY 9

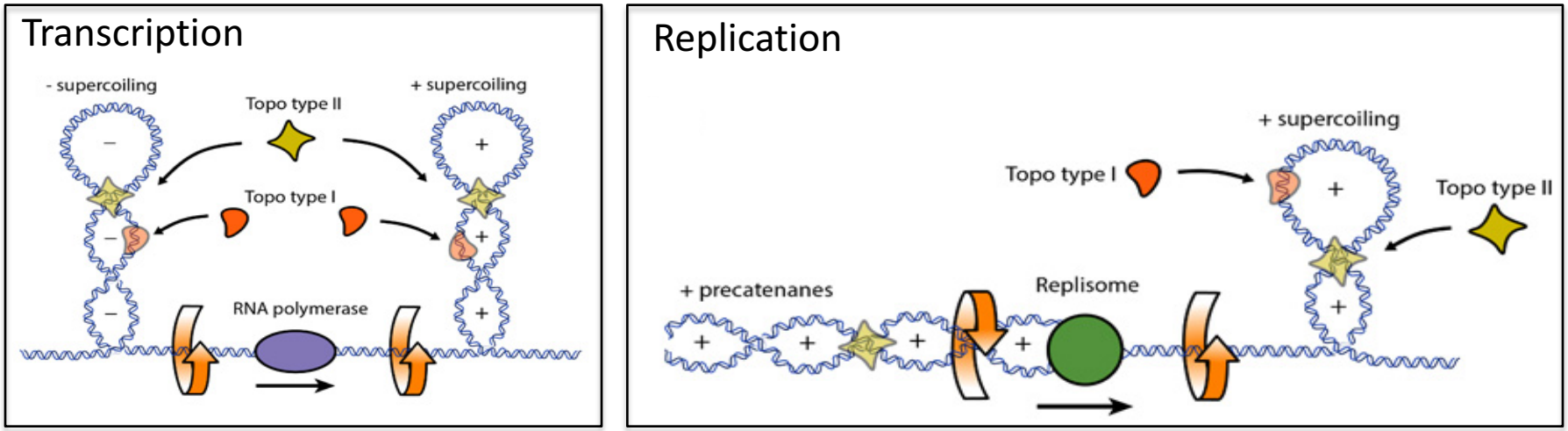


measure cell survival

Etoposide is a drug/chemotherapy that causes DNA double strand breaks

- Mechanism of action: forms ternary complex with DNA and topoisomerase II enzyme, prevents re-ligation of the DNA strands
- Cancer cells (quickly dividing cells) rely on topoisomerase II more than normal cells

Topo Type II = topoisomerase II enzyme



Ma, J. & Wang, M.D. Biophys Rev (2016) 8(Suppl 1): 75. https://doi.org/10.1007/s12551-016-0215-9

Measuring synthetic lethality in our parental and BRCA2-/- cell line

What is synthetic lethality?

combination of deficiencies (eg. knockout, LOF, drug) in 2 or more genes resulting in cell death. one deficiency alone does not cause death

What parallel pathways are we perturbing in this experiment?

DSB → HR (BRCA2-/-)

DSB → NHEJ (Loperamide, Mibifradil)

What is the result we will assess?

cell viability: cell titer glo - measures ATP via luminescence

Six compounds identified that target NHEJ and / or HR

1 = DMSO, no effect

0 = total loss of activity

	Repair activity	
Drug name	**NHEJ**	**HR**
Pimozide	0.28	0.55
→ Loperamide	0.20	0.57
→ Mibefradil	0.28	0.57
Etoposide	0.65	0.08
SR 59230A	0.27	0.58
AMN082	0.19	0.92

- Loperamide = slows contractions of intestines, treatment for gastrointestinal ailments
- Mibefradil = blocks calcium channels, treatment for heart conditions

(Slide from Noreen's Lecture 4/5)

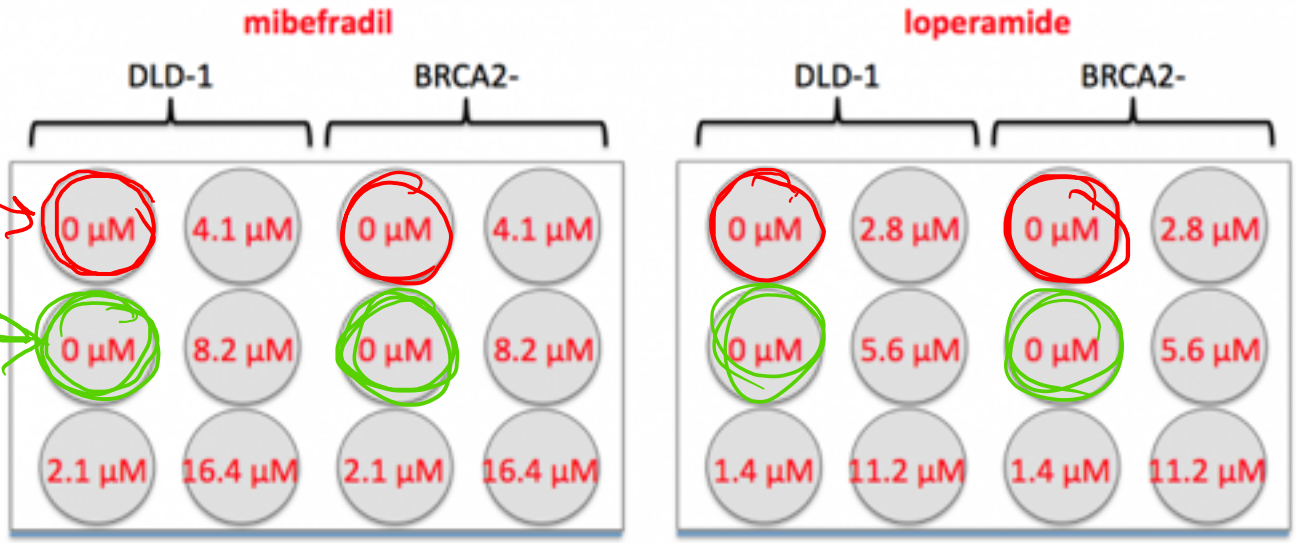
Goglia et al. (2014) *Molecular Cancer Therapeutics* 14:326-342

Synthetic lethality part 1: experiment overview

1. Choose miberfradil or loperamide, sign up at front bench
2. Induce double strand breaks (etoposide 37°C for 60min)
3. Remove etoposide media and incubate with appropriate concentration of miberfradil or loperamide till M2D9

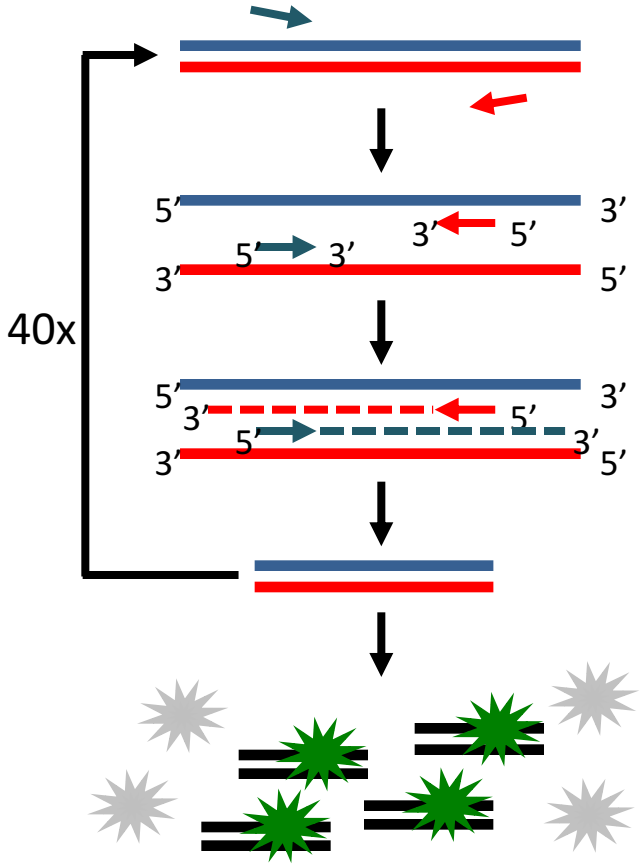
No etoposide!
No mibefradil/loperamide

Yes etoposide
No mibefradil/loperamide



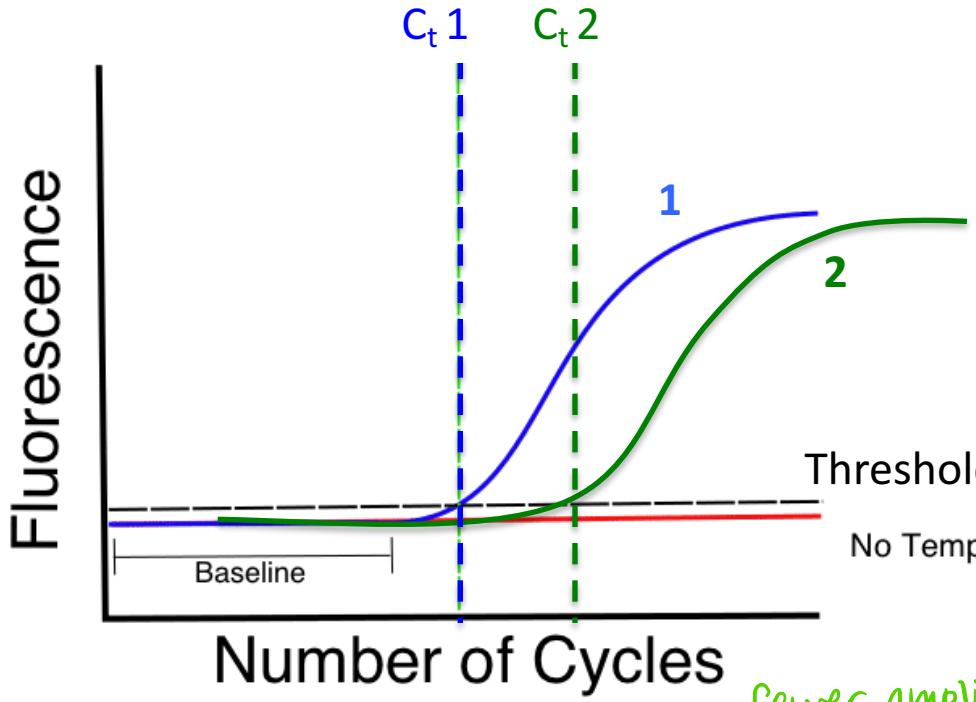
qPCR (quantitative PCR) is used to detect and quantitate gene expression

- Fluorescence is a function of dsDNA concentration via SYBR green dye
- Initial DNA concentrations are proportional to RNA purified from cells (from which we made cDNA)
- We can compare expression of a particular gene in different conditions by measuring the abundance of the gene-specific transcript
- Expression of the gene of interest is normalized to a housekeeping gene, GAPDH



Calculate relative amounts of cDNA based on threshold cycle (C_T)

Fluorescence ∝ [dsDNA]



- CT is calculated from qPCR after all cycles complete
- Which gene has higher expression—that represented by Curve 1 or Curve 2?

① $Ct_1 < Ct_2$

initial $[cDNA]_{①} >$ initial $[cDNA]_{②}$

fewer amplification cycles to reach same level of fluorescence

https://bitesizebio.com/24581/what-is-a-ct-value/

Calculate relative amounts of cDNA based on threshold cycle (C_T)

Goal: Find relative expression of p21 to GAPDH = $\frac{[\text{p21 mRNA}]}{[\text{GAPDH mRNA}]} = \frac{[\text{p21 cDNA}]_0}{[\text{GAPDH cDNA}]_0}$

initial

cDNA concentration

Assuming perfect exponential amplification during each cycle of PCR:

$[p21] = [p21]_0 * 2^{\text{Cycle\#}}$

$[GAPDH] = [GAPDH]_0 * 2^{\text{Cycle\#}}$

- Fluorescence ∝ [dsDNA]
- @ C_T, ≈ fixed [dsDNA] assuming similar lengths

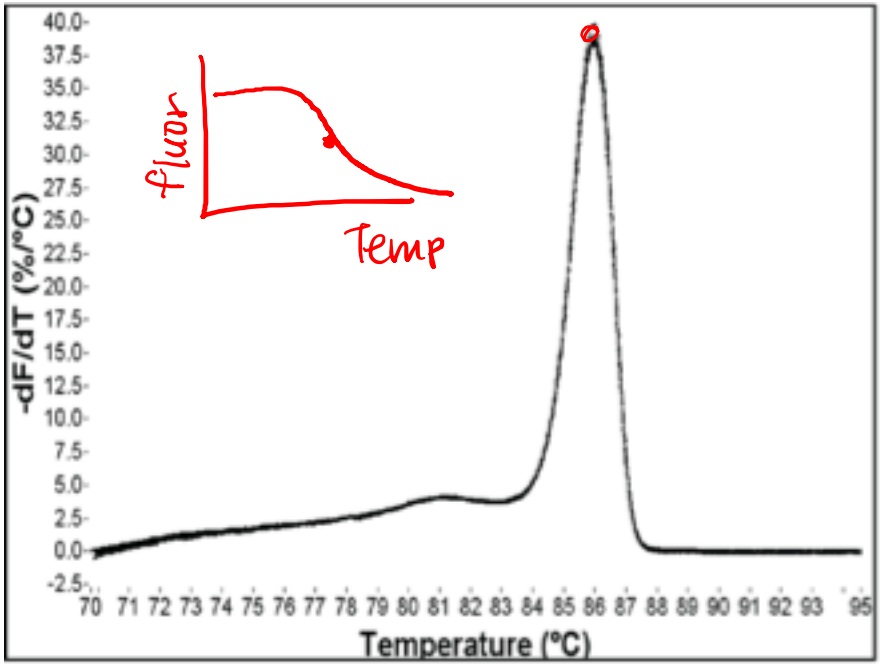
at C_T, $[p21]|_{C_{T\,p21}} = [GAPDH]|_{C_{T\,GAPDH}}$

$[p21]_0 \times 2^{C_{T\,p21}} = [GAPDH]_0 \times 2^{C_{T\,GAPDH}}$

$$\frac{[\text{p21 cDNA}]_0}{[\text{GAPDH cDNA}]_0} = 2^{(C_{T\,GAPDH} - C_{T\,p21})} = 2^{-(C_{T\,p21} - C_{T\,GAPDH})}$$

qPCR melt curve indicates the number of dsDNA products in reaction

Negative derivative of fluorescence vs. temperature



What would cause multiple peaks?

multiple products

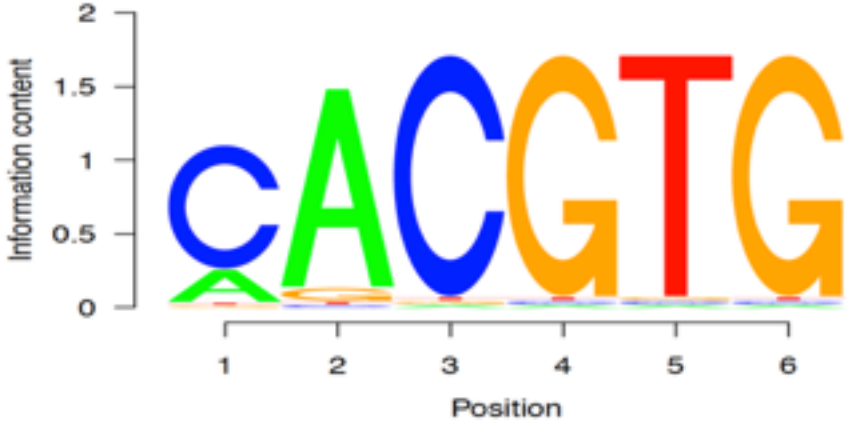
↳ primers created off-target product, sequence similar

→ accidentally put multiple primer pairs for different genes in same well

→ splice variants

Computational exercise—transcription factor binding site motifs

- Calculate position weight matrices
- Search public database of transcription factor binding
- Scan sequences to look for matching motifs
- Practice expectation-maximization algorithm for de novo motif discovery



Today in lab

1. Drug treat cells in tissue culture:
   - 1st: Pink, Purple, Platinum
   - 2nd:Red, Orange, Green, Blue
2. Analyze qPCR data
3. Complete “Transcription Factor Motifs” R exercise

HW due M2D9:

- Create figure of qPCR analysis with related RNA seq data (plot of p21 expression in various conditions), including figure title and caption
- Associated results section
- Associated discussion section