# M2D7: Perform quantitative PCR experiment and explore additional RNA-seq dataset

1. Email distribute Quiz, due on Stellar at 10pm
2. Prelab discussion
3. Review qPCR experiment
4. Statistical analysis exercise
5. Continue working on R.studio.cloud Ex3

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# Mod2 major assignments

- **Research Article (20%)**
  - individual, submit on Stellar
  - due Monday April 20th at 10pm
  - format: word document, figures can be submitted separately
- **Journal Club Presentation (17.5%)**
  - presentation **slides** due on Stellar April 11th 10pm
  - Presentation **video** due to Dropbox April 11th 10pm (details to follow)
  - format: powerpoint or pdf
- Lab quizzes M2D7, M2D9
- Homework and Notebook (10%)
- Blog (5%), 3 posts for full credit
  - 4/6 at 10 pm, 4/13 at 10 pm, 4/21 at 10 pm, 5/12 at 10pm

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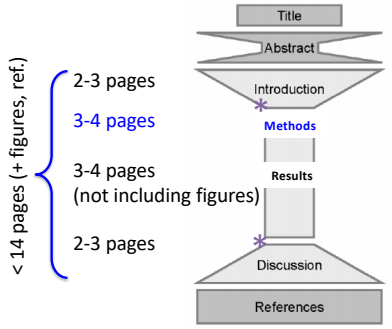
# Mod2 Research Report (20% of final grade)




- Title, Abstract (10%)
- Introduction (10%)
- Methods (20%)
- Results, Figures and captions (50%)
  - -Top 5 Up GO terms, top 5 Down GO terms (Ex2)
  - - qPCR
  - -RNA seq plots from Ex3 and Ex4
- Discussion (10%)
- References (not included in page count)

* Note on organization: last paragraph of introduction and first paragraph of discussion reiterate similar message

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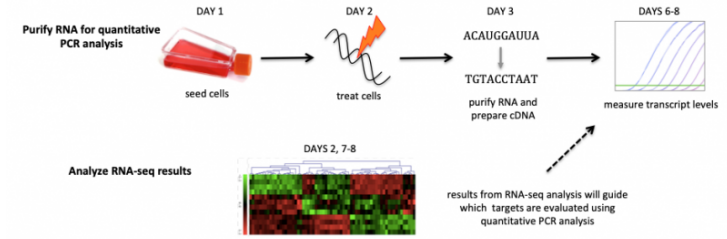
# Mod2: Experimental overview

What genes are differentially expressed in response to etoposide treatment?




Why are you using qPCR to measure individual gene expression?
How does the qPCR data relate to the RNA-seq data?

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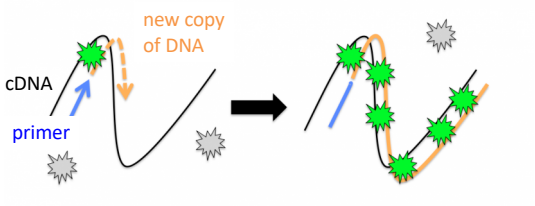
# qPCR: quantitative polymerase chain reaction

- Monitor PCR product with fluorescence
  - 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 (-> mRNA -> gene expression )

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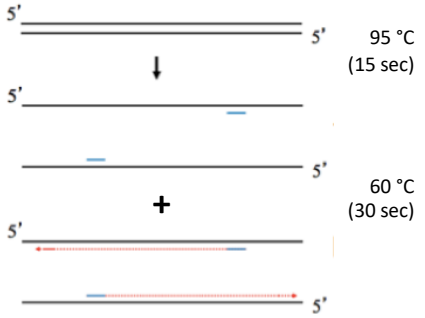
# iQ SYBR Green Supermix qPCR reagents and cycling conditions

| PCR ingredients |
|---|
| SYBR green |
| cDNA mix (template) |
| buffer and water |
| sequence-specific primers |
| iTaq DNA polymerase |
| dNTPs |

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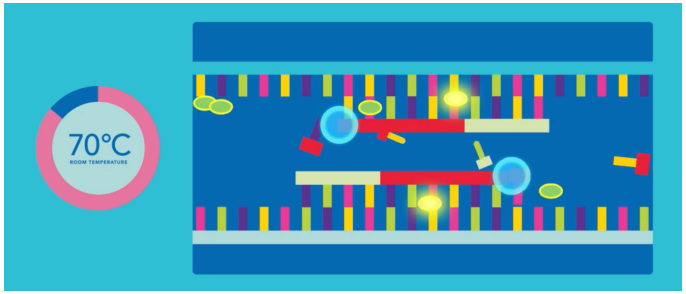
# https://www.youtube.com/watch?v=GCzH2Wcvd8E

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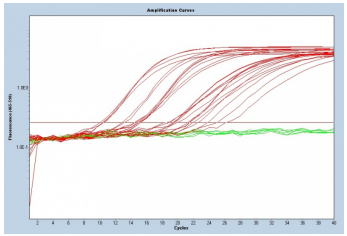
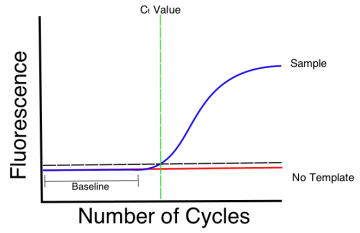
# qPCR data output is the threshold cycle ($C_T$)

- 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 cDNA






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

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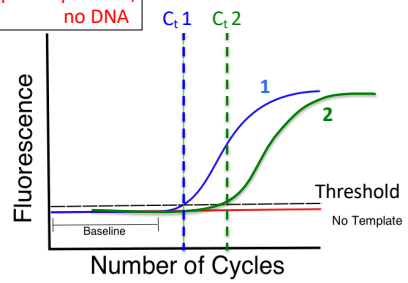
## $C_T$ related to amount of template present at the start of the amplification reaction

Blue: qPCR rxn 1
Green: qPCR rxn 2
No template: qPCR rxn, no DNA




- $C_T$ is calculated from qPCR after all cycles complete
- If $C_t$ 1=18 and $C_t$ 2=22 which reaction has a higher signal?

qPCR reaction 1, fewer amplification cycles needed to cross the threshold for background fluorescence

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

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## Practically-- Calculating Δ $C_T$ for a gene

- The analysis output of a qPCR reaction is a $C_T$ value
  - You can not directly compare $C_T$ values of samples due to variation between qPCR reactions and experiments
  - In the excel sheet there are three $C_T$ values for each gene that represent three triplicate wells in the experiment
  - The three tabs in the excel represent 3 experiments carried out on different days
- You must normalize your $C_T$ to a gene you know should not change between your samples and in response to treatment
  - We chose the abundant housekeeping gene TBP, TATA binding protein
  - You need to use the TBP $C_T$ values in the experiment you chose to calculate (TBP on the same tab of the excel)
- Finally exponentially transform each normalized value to the ΔCT expression

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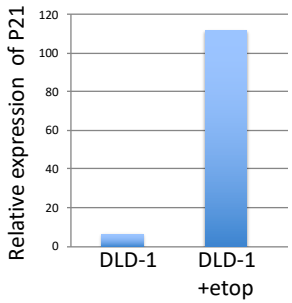
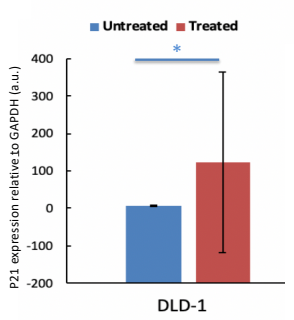
## How will you use statistics to analyze your qPCR data?

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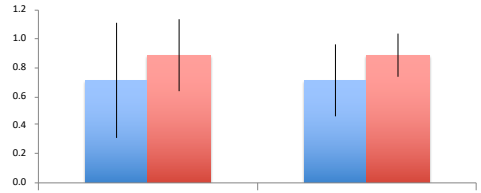
## Confidence intervals show the variance in the data set

- Assumes data follow a t-distribution
- At 95% confidence interval, there is a 95% chance that the true mean is within the defined range when n (# samples) is small




Error bars depict: 95% CI Standard Deviation

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## Calculating Confidence interval in excel

= CONFIDENCE.t(confidence level, standard dev., size)

0.05

Need to calculate standard deviation in separate cell =STDEV

n

Once you have calculated the confidence interval you will enter this value as your "custom" error bar in excel

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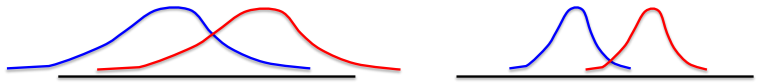
## Student's *t*-test used to determine if populations are significantly different

- Assume data follows *t*-distribution
- At $p < 0.05$, there is less than a 5% chance that populations are the same (95% chance that populations are different)
- Examines signal (means):noise (variance) ratio



high noise

low noise

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## Calculating Student's *t* in excel

*p = TTEST (array1, array2,2,3)*

Use the fewest assumptions: two-tailed

unequal variance

Can only compare two data sets at a time

*Make sure it is clear on your plots/writing which conditions are being compared

Use the p value to determine if two two conditions are significantly different or not different

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## M2D7 "Lab" Checklist

1. Watch qPCR video at the top of the Protocol section and read through Part 1.
2. Calculate the $\Delta C_T$ values for the 2-3 genes you would like to investigate further
3. Practice calculating confidence intervals and p-values with the Student's t-test
   - We suggest practicing using the $\Delta C_T$ values you will use for your research article
4. Continue working on R studio cloud Ex3.
   - We will send out code to assist with the refresher exercise today

- Homework due M2D8: Peer review methods
  - We will email you another students methods today
  - You should comment on the methods similarly to instructor's feedback – add a number to the place you'd like to comment and submit a separate document with comments
  - There are overview questions in the homework prompt you should address at the bottom of your specific comments

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