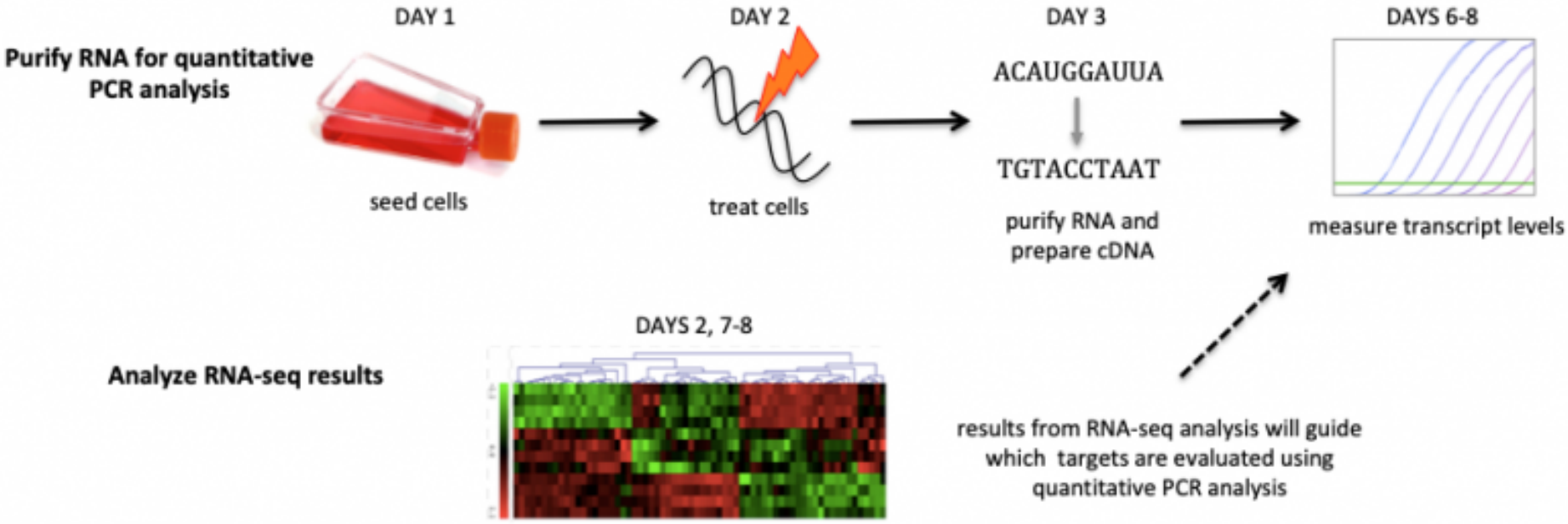
M2D3: Purify RNA and practice RNA-seq data analysis methods

See my notes in red. Please feel free to email me for any further clarification!

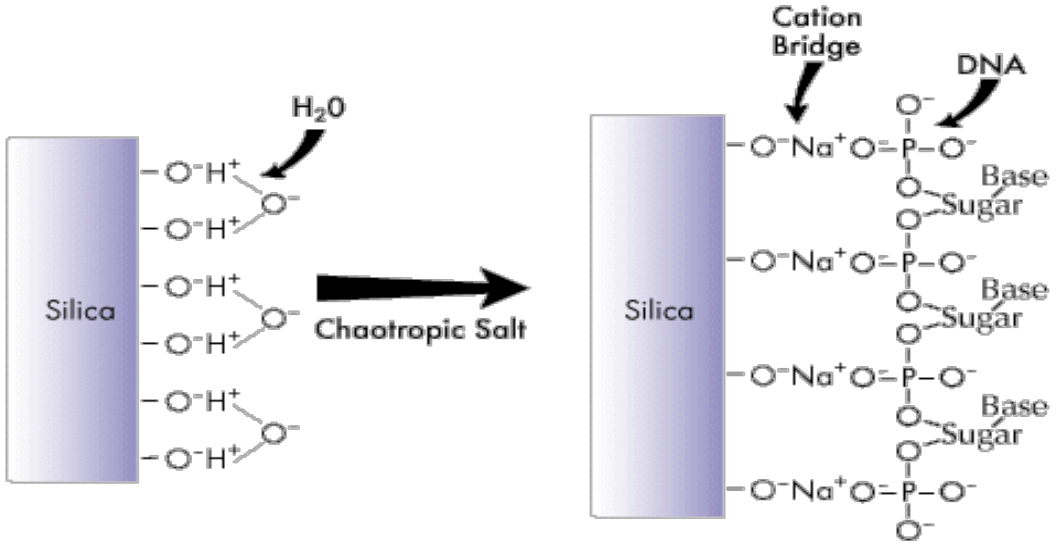
Mod2: Experimental overview



Isolate RNA: QIAshredder + Rneasy kit

steps	contents	purpose
lyse	RLT (with highly denaturing guanidine-thiocyanate salt)	Lysis buffer with chaotropic salts (i.e. guanidine-thiocyanate salt) disrupts hydrogen bonds of water and hydrophobic interactions of proteins, but not DNA or RNA
lyse	QIAshredder (purple column)	Shears membranes to release RNA and improve extraction
prepare	70% ethanol	Promotes binding of RNA to silica column (see next slides for details)
bind	silica membrane (pink column)	Binds RNA backbone to extract it from the solution (optimized for mRNA)
wash	RW1 RPE	Washes away molecules not bound to the silica ** after this wash, important to get rid of *all* ethanol (ethanol in the buffers)
elute	water, RNase-free	Elutes RNA from silica (see next slides for details)

Chaotropic salts and Ethanol help DNA/RNA bind to column

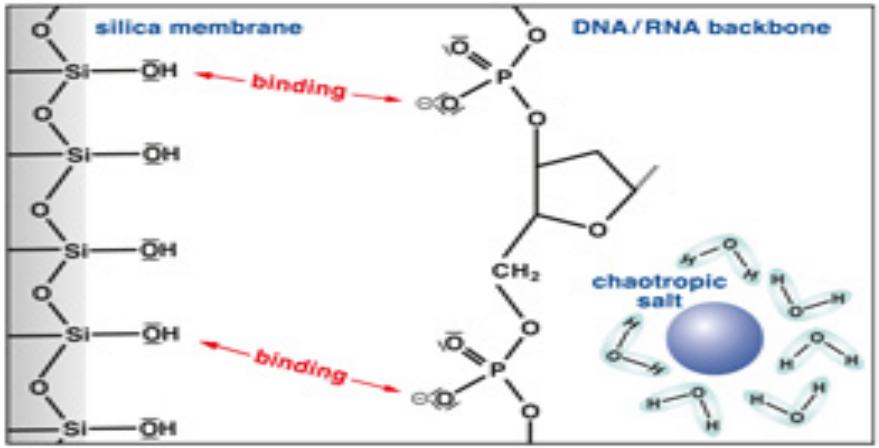


EtOH and chaotropic salts disrupt hydrogen bonds of water to promote binding of the RNA backbone to the silica

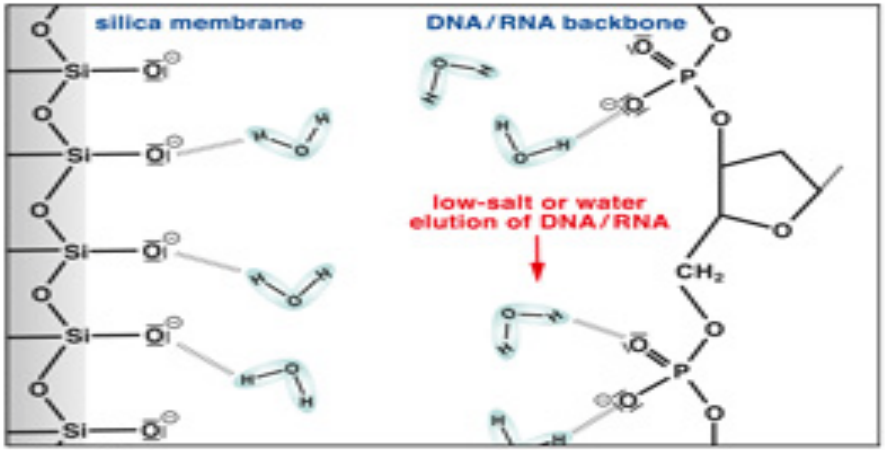
- Washes with RW1 and RPE remove residual contaminants
  - RW1 contains a guanidine salt, as well as ethanol, and is used as a stringent washing buffer that efficiently removes biomolecules such as carbohydrates, proteins, fatty acids, etc, that are non-specifically bound to the silica membrane
  - RPE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

Water is used to elute RNA from column

before elution



after elution

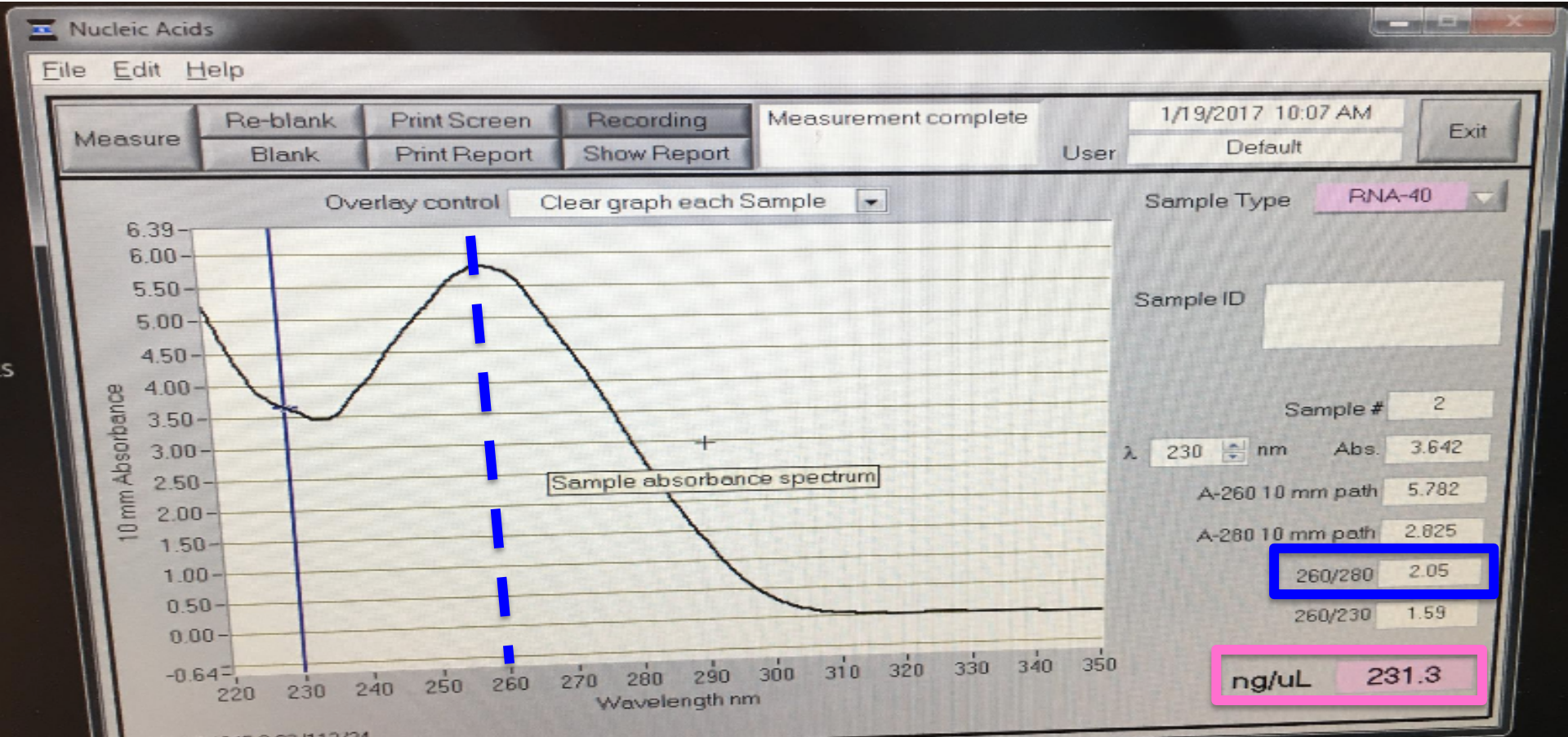


- Flooding the column with water competes mRNA off the column to elute

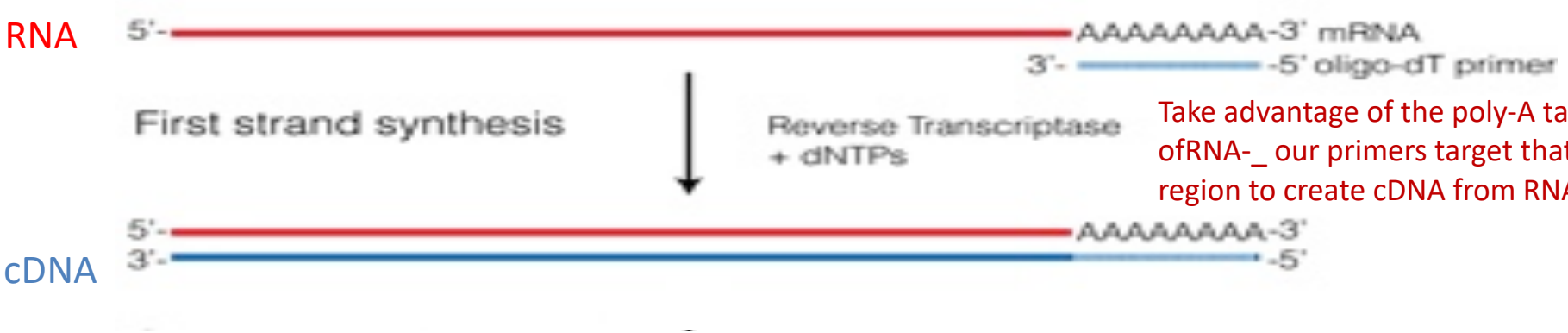
Determine RNA concentration and purify from NanoDrop spectrophotometer

- A_{260}/A_{280}
  - nucleic acids absorb at nm
  - proteins absorb at nm
  - ratio ~ 1.8 “pure” DNA
  - ratio ~ 2.0 “pure” RNA

RNA concentration and purify from NanoDrop



Reverse Transcriptase Reaction: Utilizing the poly-A tail to synthesize cDNA from purified RNA



Note: Once we have purified mRNA we want to convert it to cDNA and use that in our qPCR experiment. To do this, we use a reverse transcriptase enzymatic reaction (think about how HIV works) to convert RNA to cDNA.

- Why synthesize cDNA? cDNA is far more stable to work with than mRNA
- Reverse transcriptase PCR (RT-PCR) (described above to convert mRNA to cDNA) vs. Real time PCR (RT-PCR or qPCR) Describes amplification of specific cDNA during a PCR cycle, monitored by a fluorescent dye. Often inter-related terms, confusingly giving the same acronym.

Components and procedure of cDNA Synthesis

step/purpose	conditions	reagents added
Denature & anneal / reduce secondary structure of RNA so OligoT primer can anneal to polyA tail	65°C 5 min on ice 1 min	RNA dNTPs oligoT primer
synthesize cDNA	50°C 50 min	RT enzyme $MgCl_2$ (enzyme cofactor) Buffer RNAseOut (prevent RNA degradation)
Terminate / stop reverse transcriptase enzyme	85°C 5 min	High heat to denature enzyme
Remove RNA / only cDNA remaining	37°C 20 min	RNase H

What genes are differentially expressed in response to DNA damage?

How are we addressing this question?

DLD-1 cells: Compare 2 groups

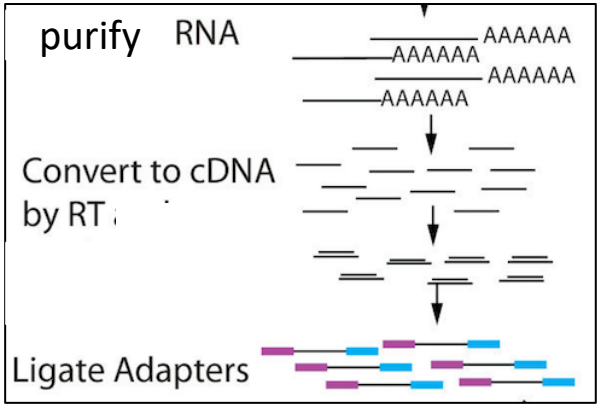
- No Treatment
- 60min Etoposide

We are using 2 methods to compare mRNA expression in the drug-treated vs control cells

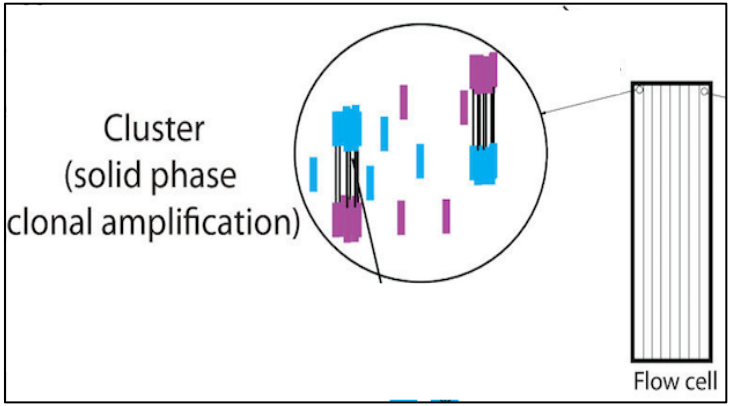
1. High throughput Illumina RNA-seq
    1. Assess all mRNA/cDNA present in sample
    2. Analyzed through DESeq2 in the R environment
2. qPCR with SYBR green
    1. Design primers against cDNA of gene of interest
    2. Determine expression compared to housekeeping gene

Workflow for Illumina HiSeq 2000

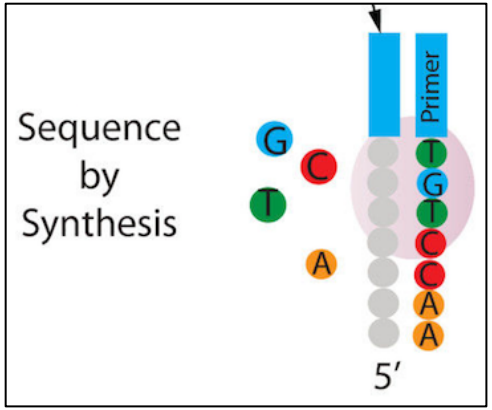
(1) sample prep



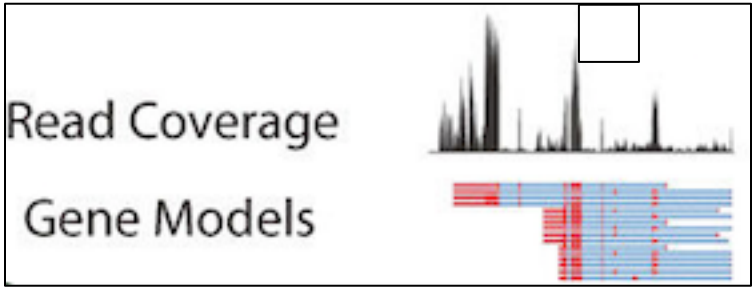
(2) amplification



(3) sequencing



(4) data analysis



Reminder of Noreen's lecture on RNA-seq

- Purify and convert RNA (like in lab)
- Add adaptors then amplify cDNA
- Sequence cDNA and map to genome

Malone JH, Oliver B, BMC Biol. 2011