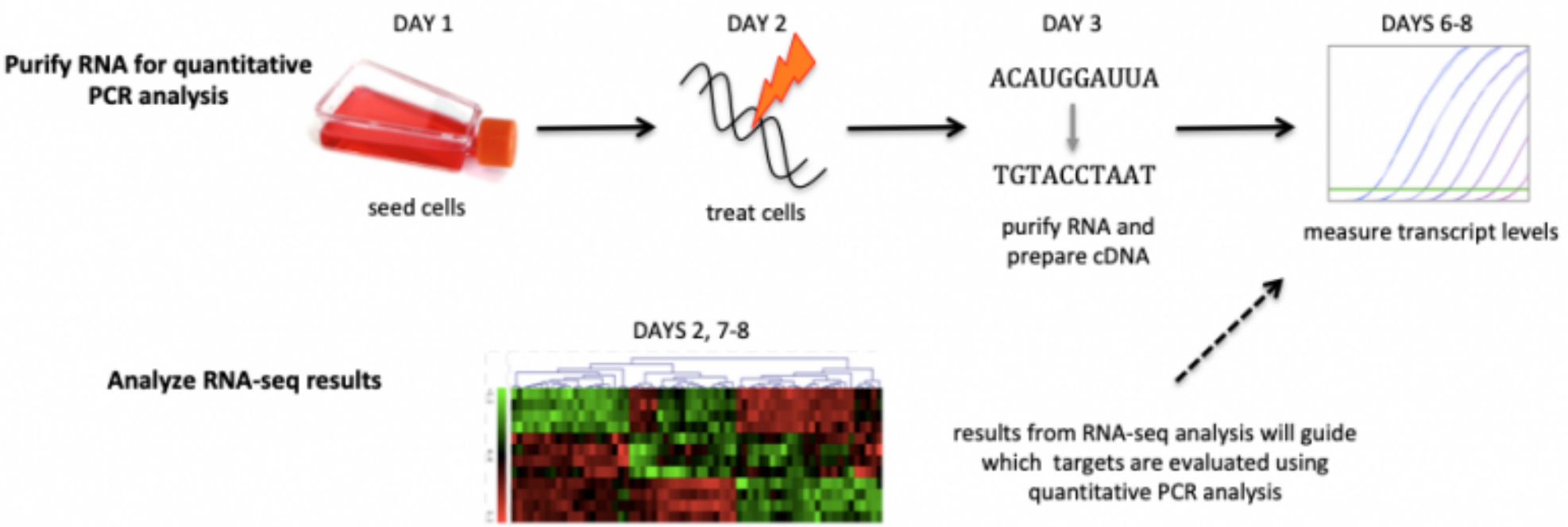
M2D3: Purify RNA and practice RNA-seq data analysis methods

1. Prelab discussion
2. Tissue culture room to harvest cells for RNA purification followed by cDNA synthesis

Mod2: Experimental overview

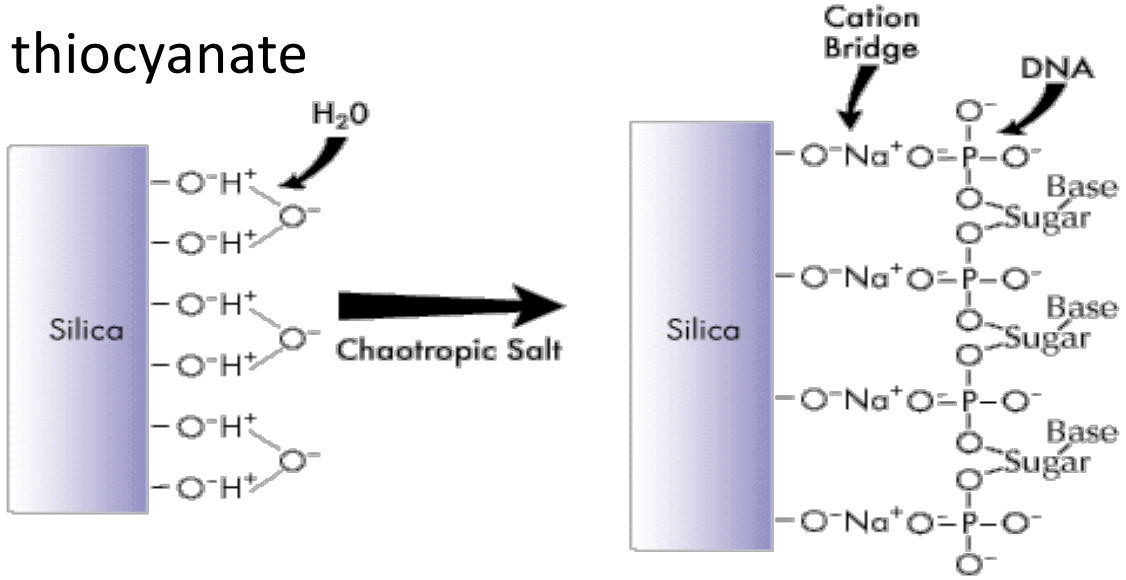


Isolate RNA: QIAshredder + Rneasy kit

steps	Buffer / column	General Contents	Purpose
lyse	RLT (with highly denaturing guanidine-thiocyanate = chaotropic salt)	Chaotropic salts detergents	Disrupt hydrogen bonding = denature proteins and lyse membrane
lyse	QIAshredder (purple column)	Polymer that shears high molecular weight components	Shear membrane, organelles = Release mRNA from cell
prepare	70% ethanol		Ethanol + salts weakens hydrophobic effects = increases likelihood RNA binds to silica
bind	silica membrane (pink column)	porous glass	Bind RNA backbone, optimized for longer than 200 nucelotide RNA (mRNA)
wash	RW1 (stringent wash) RPE (mild wash)	Chaotropic salts ethanol	Removes biomolecules not bound to membrane ** after this wash, important to get rid of *all* ethanol (ethanol in the buffers)
elute	water, RNase-free		Elute RNA from silica

Chaotropic salts help DNA/RNA bind to column

e.g. guanidine thiocyanate

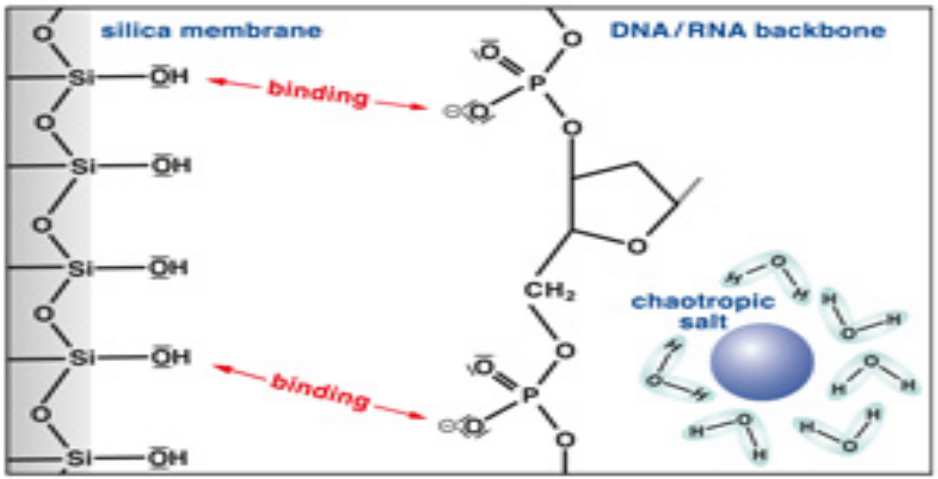


- Washes with RW1 and RPE remove residual contaminants
  - RW1 contains a guanidine salt, as well as ethanol, and is used as a stringent washing buffer that efficiently removes biomolecules such as carbohydrates, proteins, fatty acids, etc, that are non-specifically bound to the silica membrane
  - RPE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

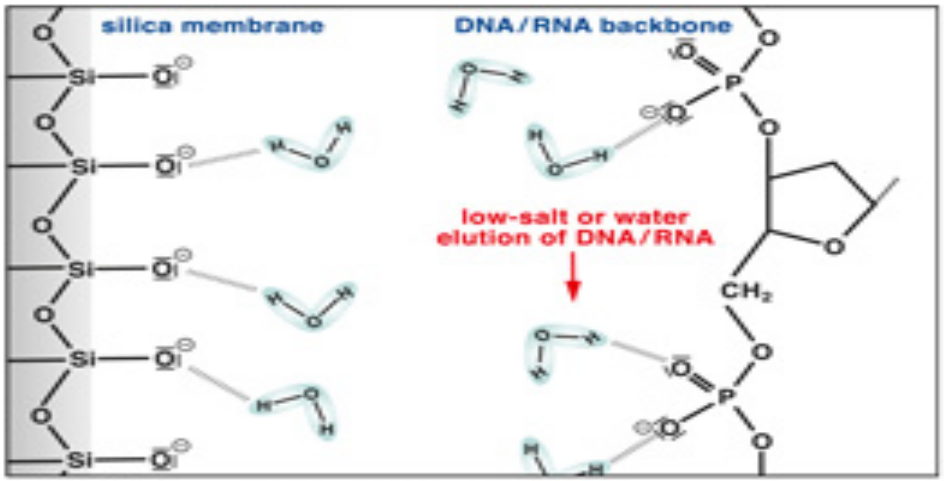
Water is used to elute nucleic acids

- Water competes RNA from column

before elution



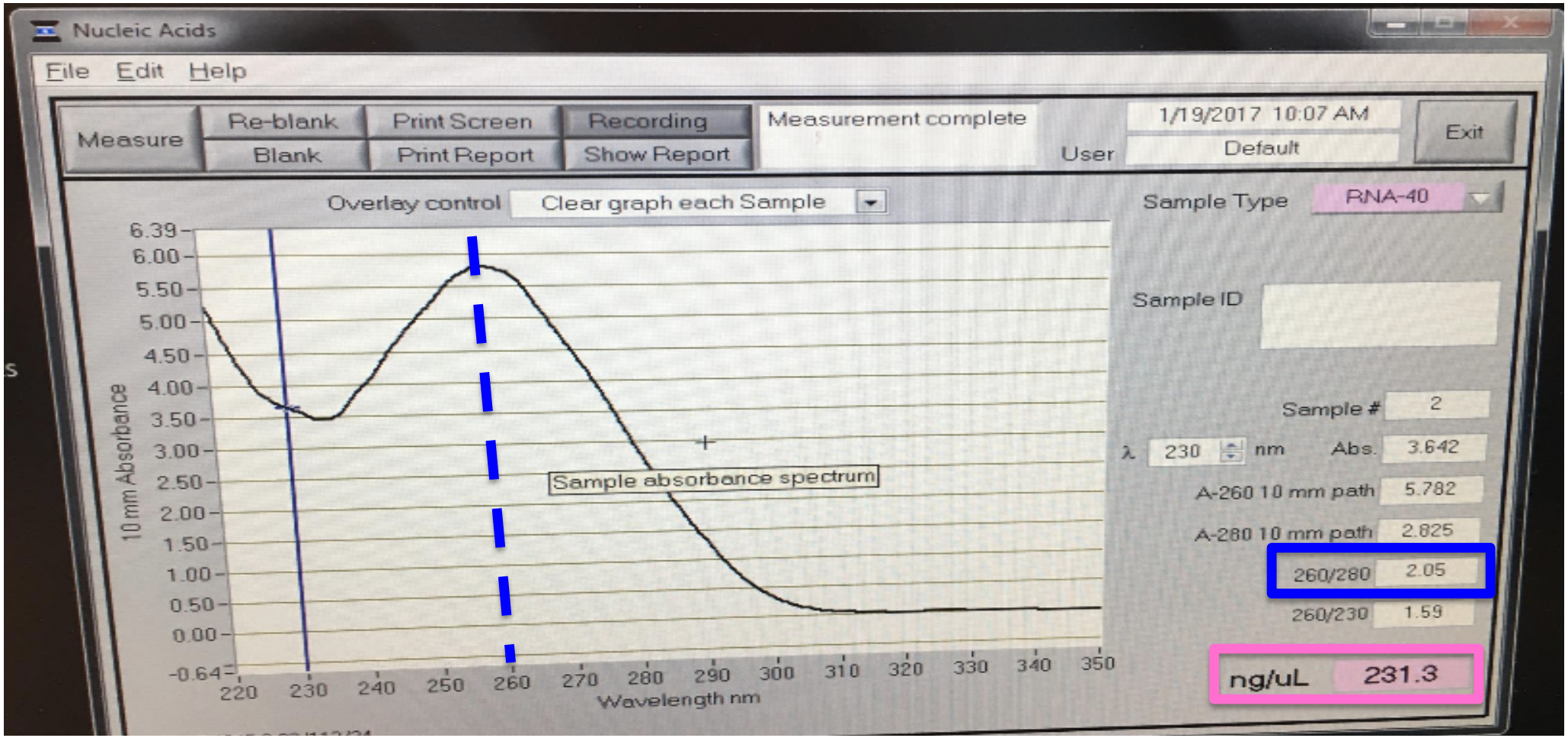
after elution



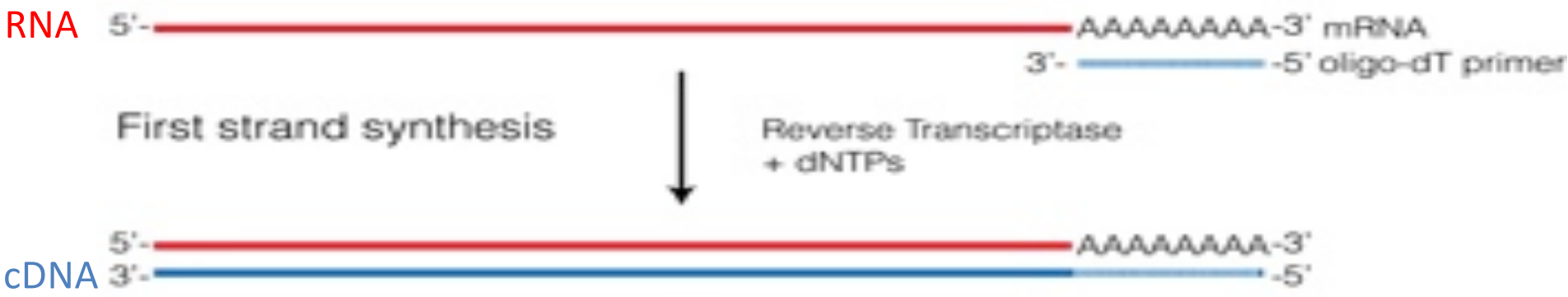
RNA concentration from NanoDrop spectrophotometer

- 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 from NanoDrop



Utilizing the poly-A tail to synthesize cDNA from purified RNA



Note:

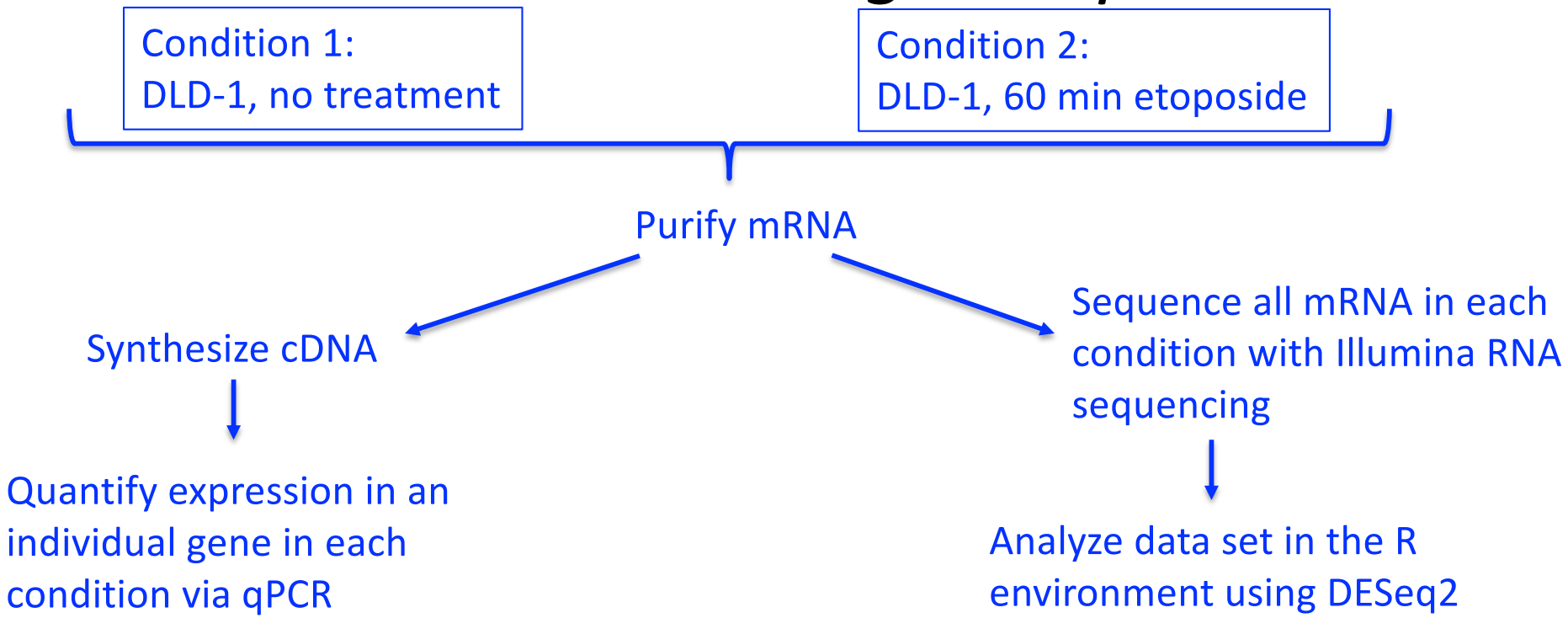
- Where was the reverse transcriptase enzyme isolated from? murine leukemia virus (RNA genome), also many retroviruses (HIV), earned 1975 Nobel prize
- Why synthesize cDNA? More stable than RNA
- Confusing acronyms~ Reverse transcriptase PCR (RT-PCR): reaction with the enzyme reverse transcriptase synthesizing cDNA from RNA vs. Real time PCR (RT-PCR or qPCR) reaction utilizes fluorescence dye to monitor and quantify amplification of a targeted DNA molecule during each cycle of PCR

Components and procedure of cDNA Synthesis

step/purpose	conditions	reagents added/purpose
Denature & anneal	65°C 5 min on ice 1 min	RNA Oligo T primer dNTPs, *Reduce secondary structure of RNA, allow primer to bind poly A tail*
synthesize cDNA	50°C 50 min	reverse transcriptase enzyme Enzyme co-factor ($MgCl_2$) and buffer RNaseOUT, *Synthesize cDNA*
terminate	85°C 5 min	Denature reverse transcriptase enzyme, *stop reaction*
remove RNA	37°C 20 min	RNase H enzyme, *remove RNA only cDNA remains*

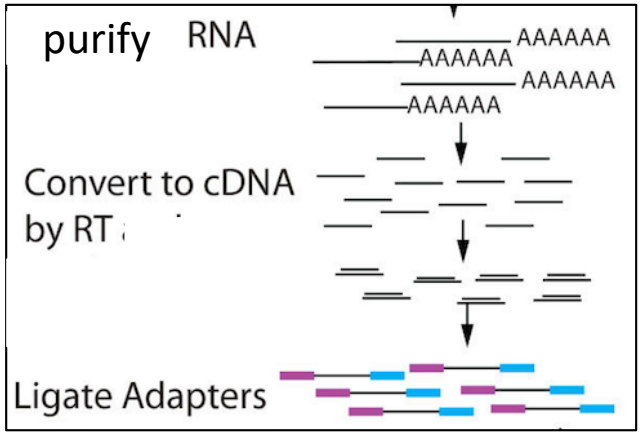
What genes are differentially expressed in response to DNA damage?

How are we addressing this question?

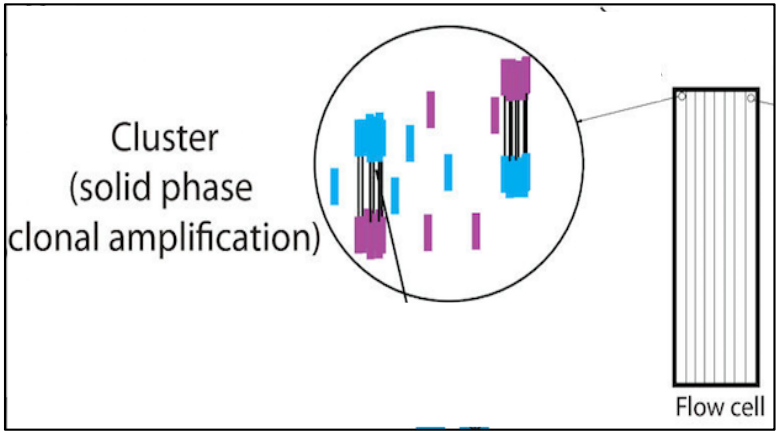


Workflow for Illumina HiSeq 2000

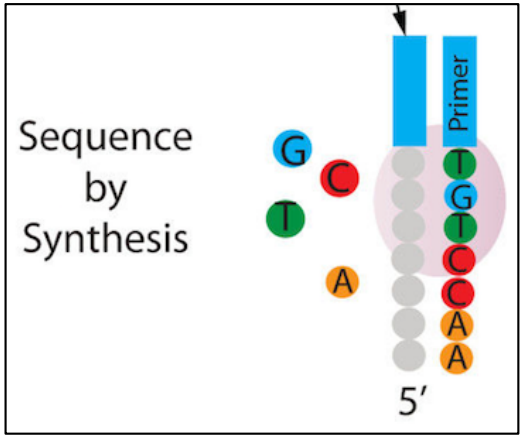
(1) sample prep



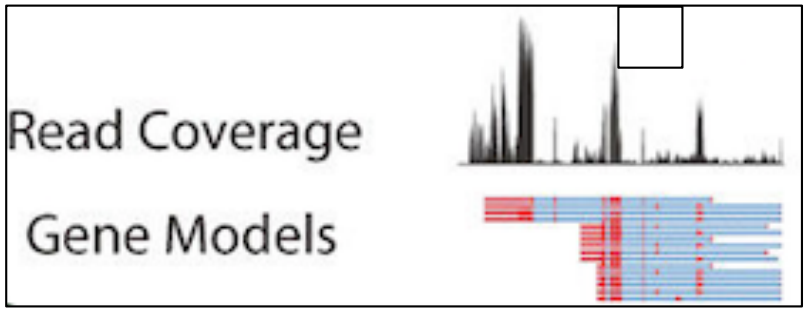
(2) amplification



(3) sequencing



(4) data analysis



Malone JH, Oliver B, BMC Biol. 2011