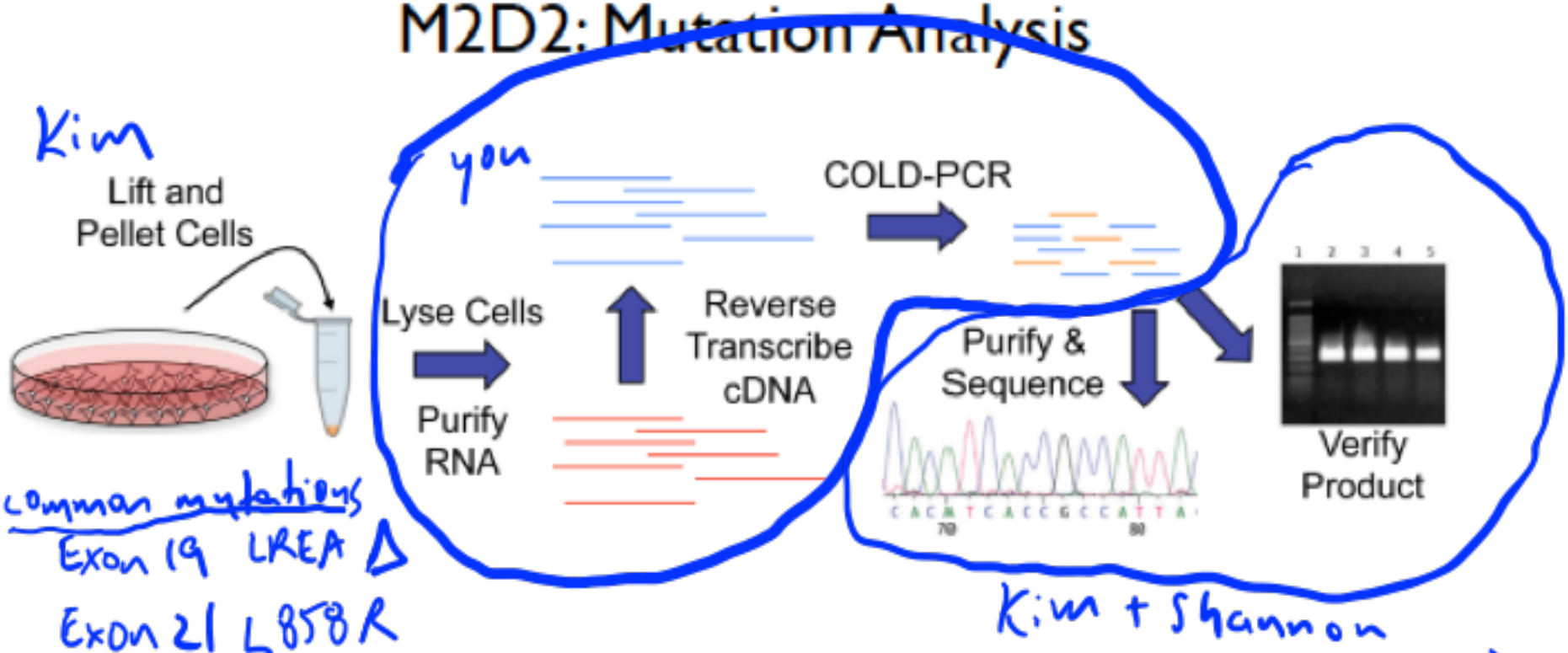
- Announcements → OH — in lab
  no lab next Wed
- Lab Quiz
  ANS 2-3
  SKH 7-8
- Pre-lab Lecture
  - D2→3 overview
  - RNA purification and RT
  - Intro to sequencing
  - COLD-PCR for mutant-hunting
  - Today in Lab: M2D2

M2D2: Mutation Analysis



you Positive Control: HCC-827 lung cancer (+/- Exon 19)

K/S Negative Control: MDA-MB231 breast " ("WT" EGFR)

you Experiment: SKOV-3 ovarian " (↑ EGFR; MUT??)

RNA Purification

unstable to RNases

- ice
- clean prep
  - gloves
  - RNase away

RLT + β-ME

viscosity ↓

QIAshredder purple! save FT!

RNeasy pink!

~100-500 K → MAX 12.5mg

~70% EtOH

nuclease-free H_2O (Tris → RT)

http://www.sabiosciences.com/pathwaymagazine/pcrhandbook/qiagen-rneasy-kits.php

Planning and carrying out RT

- Calculate [RNA] of each sample (+purity)
- Denature RNA: 70°C, 5'
- Back to ice! + master mix
- RT: 60°C, 60'

* random hexamer primers *

A1 = 40ng/mL * remember dilution vs. stock! *

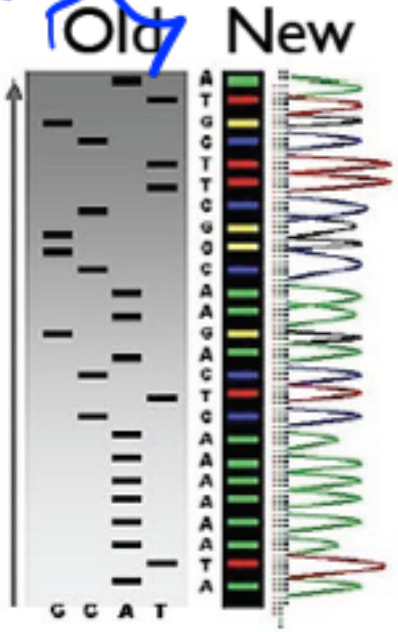
Sample	A_{260}	Measured RNA conc. (µg/mL)	Measured RNA conc. (ng/µL)	Max RNA per rxn (ng in 7.5 µL)	Volume RNA needed per rxn to obtain btn 500-1000 ng	Volume water needed per rxn (if needed)
1:	2.5	100	100	750	7.5 or scale 3.75uL + 3.75uL	
2:	1.25	50	50	375	7.5uL : C	Ø

Overview: Sanger Sequencing

Four dye labeled dideoxynucleotides added to each reaction

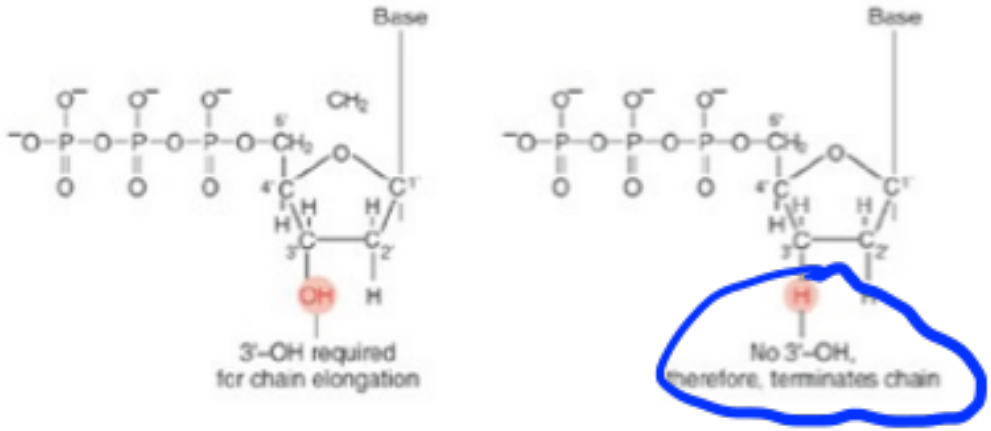
4 separate rxns.

Also add one primer (cf PCR)



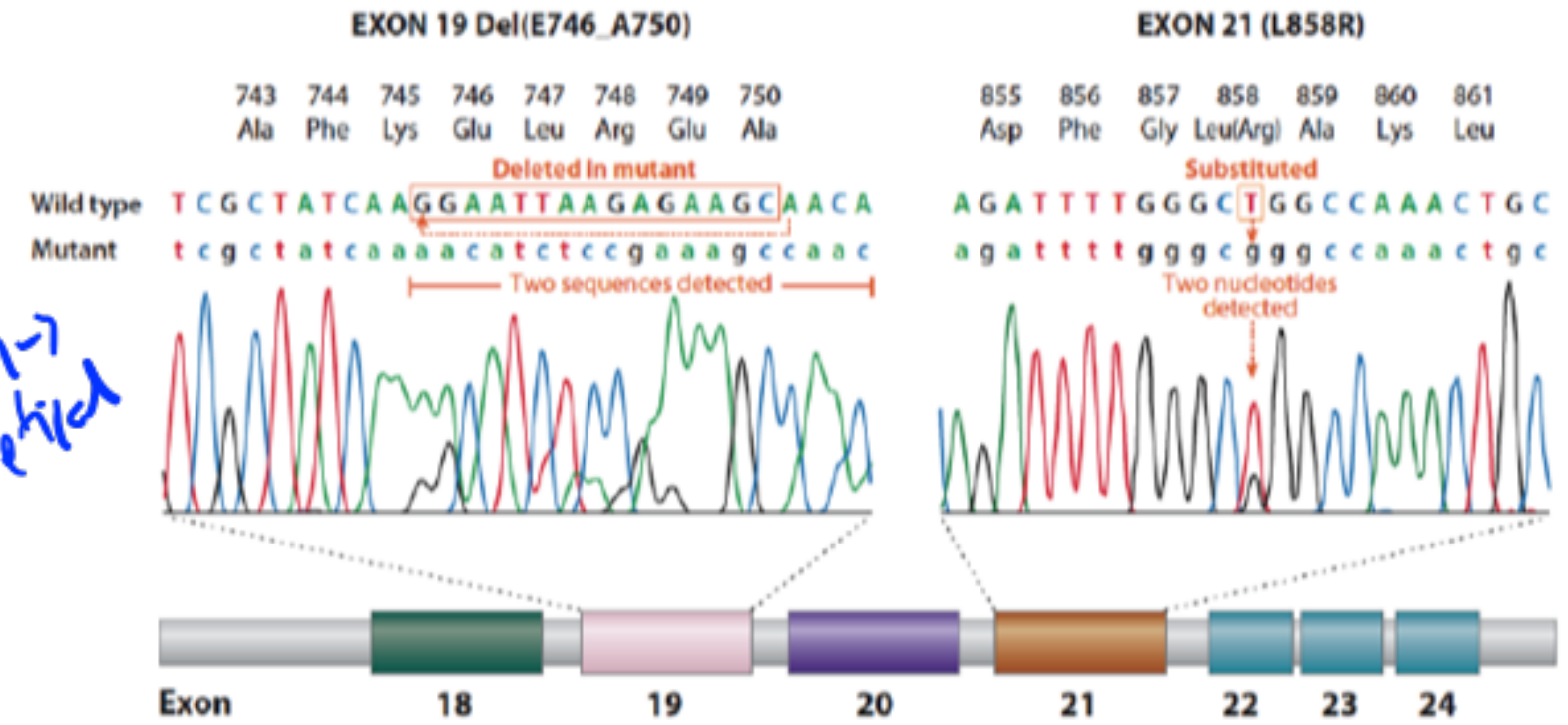
cf gel EP

cf flow cyt.



'Chain terminating reaction'

Examples of PCR-Sequencing results:



pretty→ the exception

Figure 2

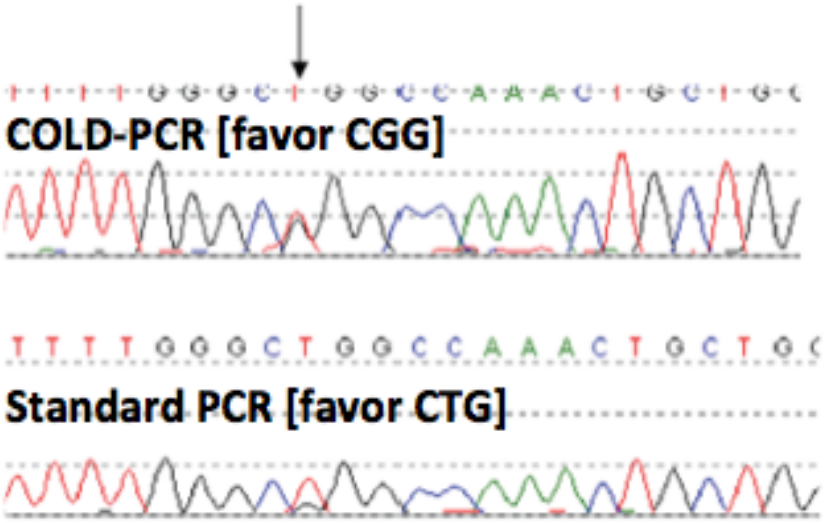
Amino acid and nucleotide sequence changes in exon 19 deletion and exon 21 L858R mutations involving the tyrosine kinase domain of epidermal growth factor receptor.

Santos et al. Annu. Rev. Pathol. Mech. Dis. 2011. 6:49–69

COLD-PCR

co-amplification at lower denaturing (Temp)

real data!



From Santis et al. (our protocol today)

Note: usually use genomic DNA for clinical samples →

- Few rounds of normal PCR, and then…
- Preferentially melt WT-mutant duplexes (not WT-WT), to make available for amplification
- 71 (cf 56): heteroduplex Ta; 87 (94): new Tm [°C]

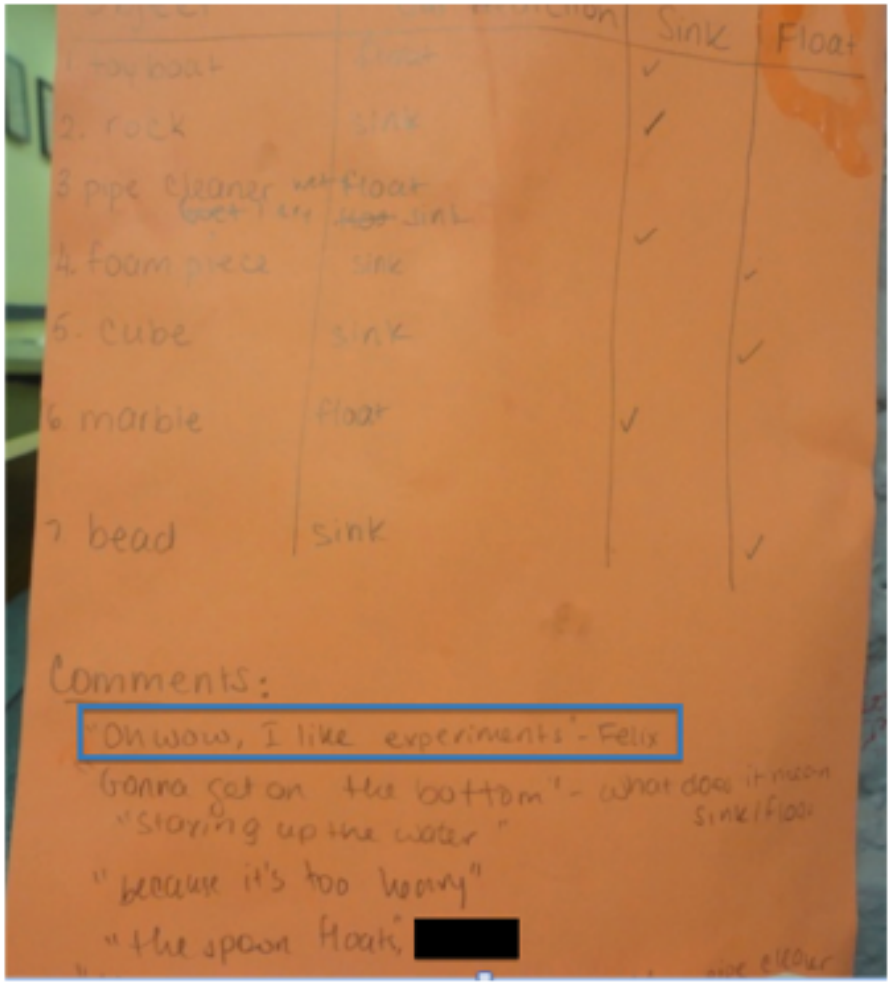
See also http://upload.wikimedia.org/wikipedia/commons/8/85/COLD-PCR.jpg

Today in Lab: M2D2

- *BL2 protocol lab coats + gloves
- *Hood protocol LC, G, glasses; close sash ✓ leave B-ME tips in there?
- RNA/RT prep → get your own ice day!
- Atissa talk on j clubs at 3:30 pm (1 h)
- PCR prep
- Next time:
  - sequencing analysis, cell prep for WB, Kirouac paper

group JC / pick Figure w/ me today

And… your toddler inspiration!



Even in the face of failure…

LJ, we have to put the human body together!

I think the moon is following us