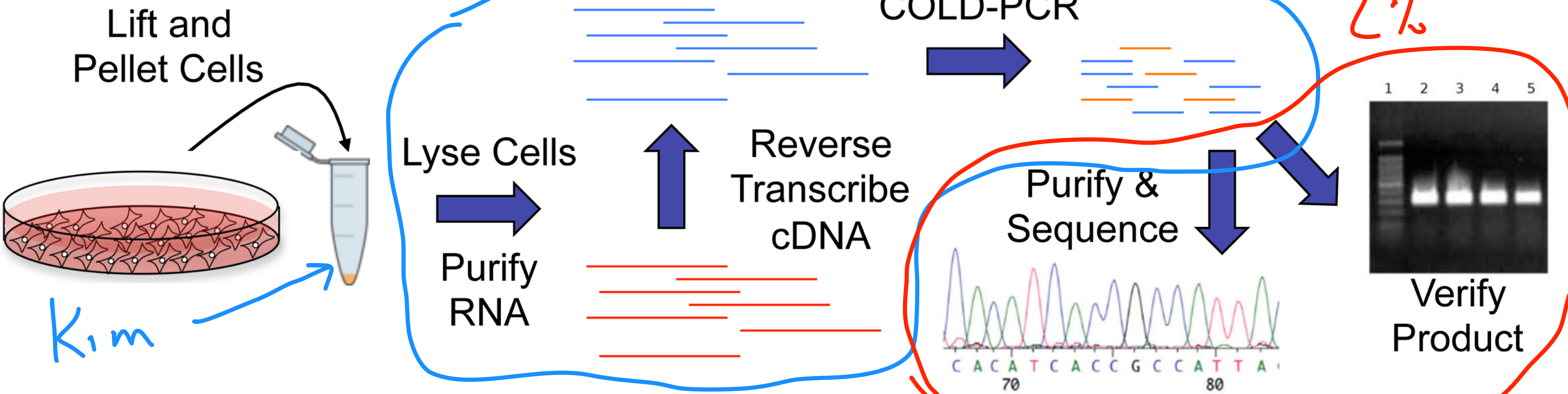
M2D2: Mutation Analysis

10/10/13

1. Lab Treat
2. Pre-lab discussion
3. RNA purification & Reverse Transcription
4. Atissa here to talk Journal Club
5. Set-up EGFR mutation PCR

*BL2 - lab coat
- gloves

M2D2: Mutation Analysis



*Exon 19 → LREA deletion
Exon 21 → L858K

Teaching staff

Positive Control: HCC-827 ← RNA (lung cancer - NSCLC)

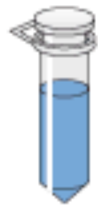
Negative Control: MDA-MB231 (breast cancer 'WT' EGFR)

Experiment: SKOV-3 ovarian cancer → ↑EGFR

RNA Purification

★ very unstable
- ice
- RNase away

Sample



Lyse, homogenize, and add ethanol → RLE (+βME) → Qiashredder (Purple)

Bind total RNA to RNeasy membrane ← RNeasy ~500K cells ~12.5μg

Wash ← 70% ethanol

Elute in small volume — 30μL RNase/DNase H_2O

Ready-to-use RNA

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

RT Reaction:

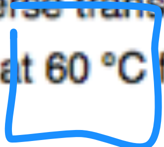
7.5 μL

Sample	A_{260}	Measured RNA conc. (μg/mL)	Minimum RNA conc. (μg/mL)	Max RNA per rxn (ng in 7.5 μL)	Volume RNA needed per rxn	Volume water needed per rxn
SKOV3 1:		100 ng/μL	50	375	3.75	3.75
HCC827 2:		50	50	375	7.5	Ø

Part 2: RT reactions

[edit]

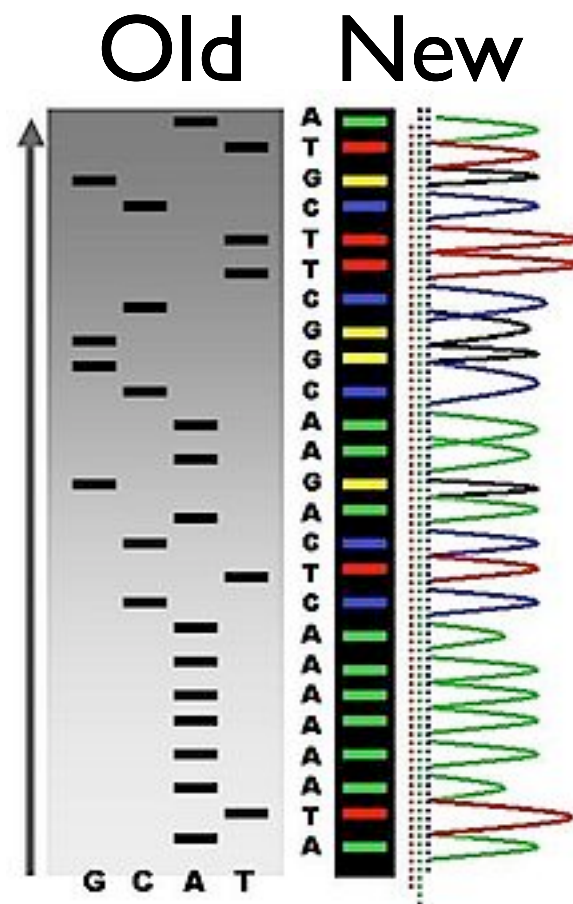
1. Set up your reactions on a cold block. You will prepare one reaction for each of your samples. Random hexamer primers will be used so that all (we hope) transcripts are amplified. This approach is more convenient than adding unique primers for each transcript of interest.
2. First, add 7.5 μL of the appropriate RNA (or RNA and water as needed) to two different PCR tubes.
3. To your RNA, add 0.75 μL random hexamers from the shared stock at the front of the room (be careful pipetting!)
4. Once everyone is ready, we will denature the RNA at 70 °C for 5 min in the thermocycler. Immediately place back on the cold block.
5. Pipet 22.5 μL of RT master mix into each of two well-labeled PCR tubes. The master mix contains water, buffer, dNTPS, primers, and reverse transcriptase.
6. The reactions will be carried out at 60 °C for 60 min in the thermocycler followed by cooling.

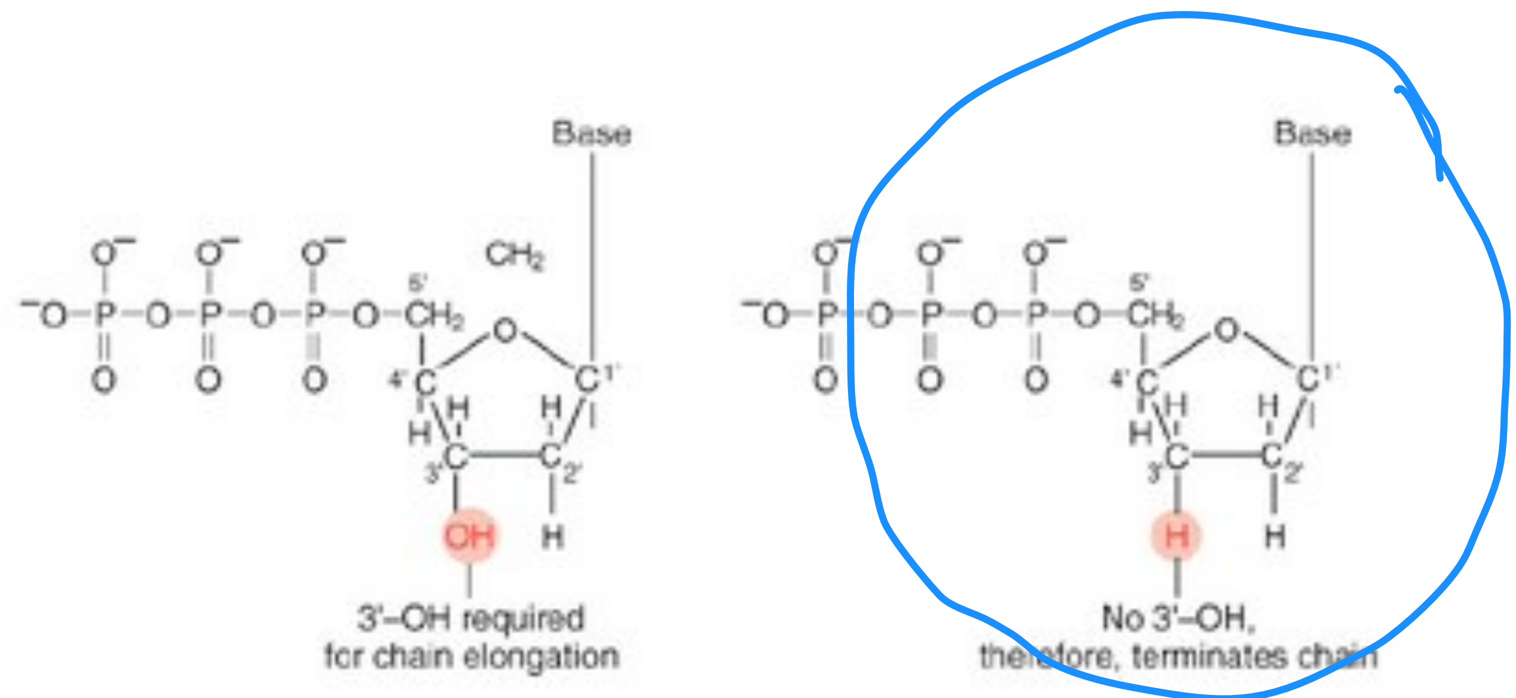


Overview: Sanger Sequencing

Four dye labeled dideoxynucleotides added to each reaction

Old New





'Chain terminating reaction'

Examples of PCR-Sequencing results:

COLD-PCR: co-amplification @ lower denaturation temp

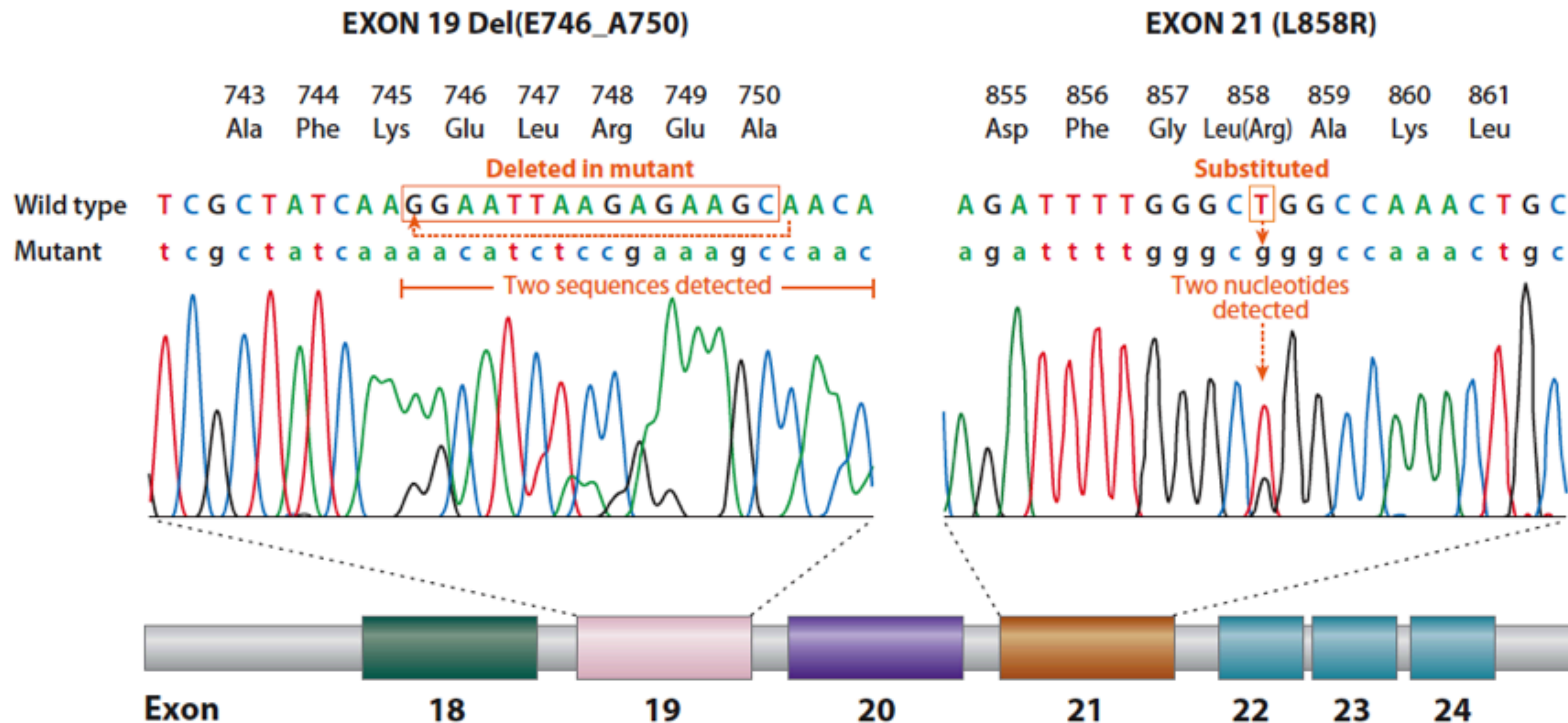
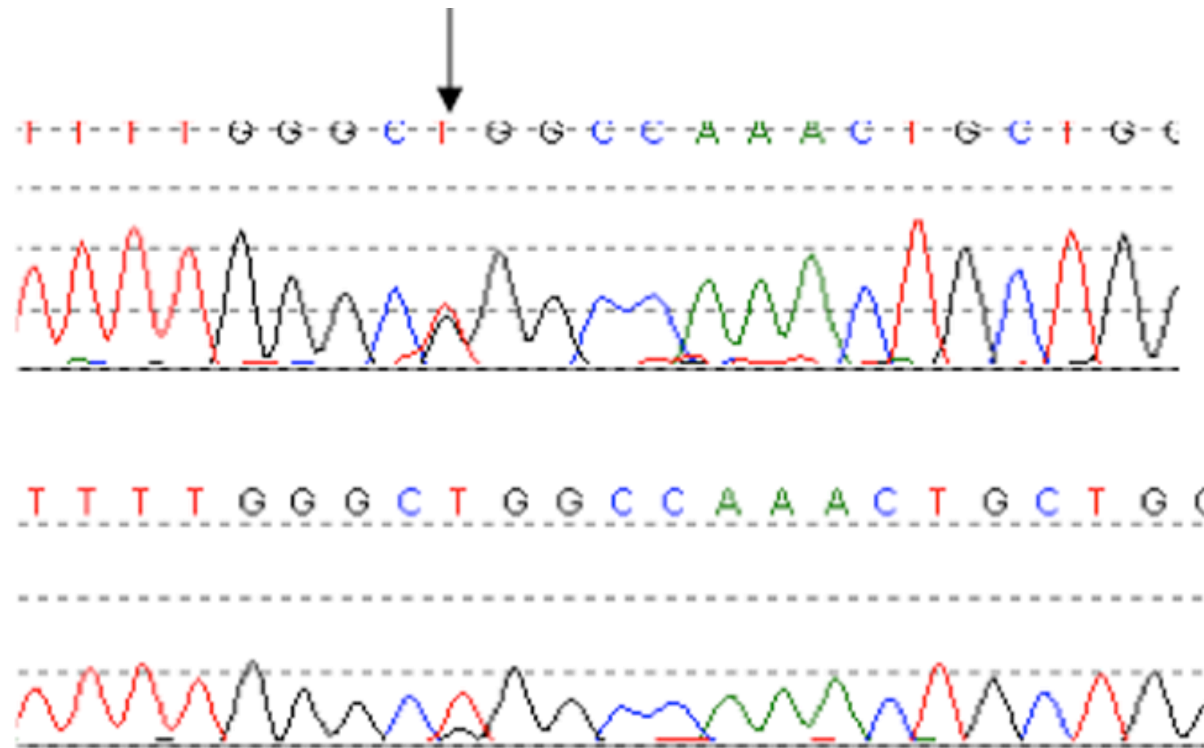


Figure 2

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



From Santis et al. (our protocol today)

COLD-PCR

EGFR L858R

Standard- PCR

. The reactions will undergo the following PCR cycle:

1. 95° 10 min
2. 94° 30 sec
3. 56° 30 sec
4. 72° 30 sec
5. repeat steps 2-4 10 times
6. 94° 20 sec
7. 71° 3.5 min
8. 87° 20 sec
9. 56° 30 sec
10. 72° 30 sec
11. repeat steps 5-9 40 times
12. 72° 5 min
13. 4° hold

Usually use genomic DNA:

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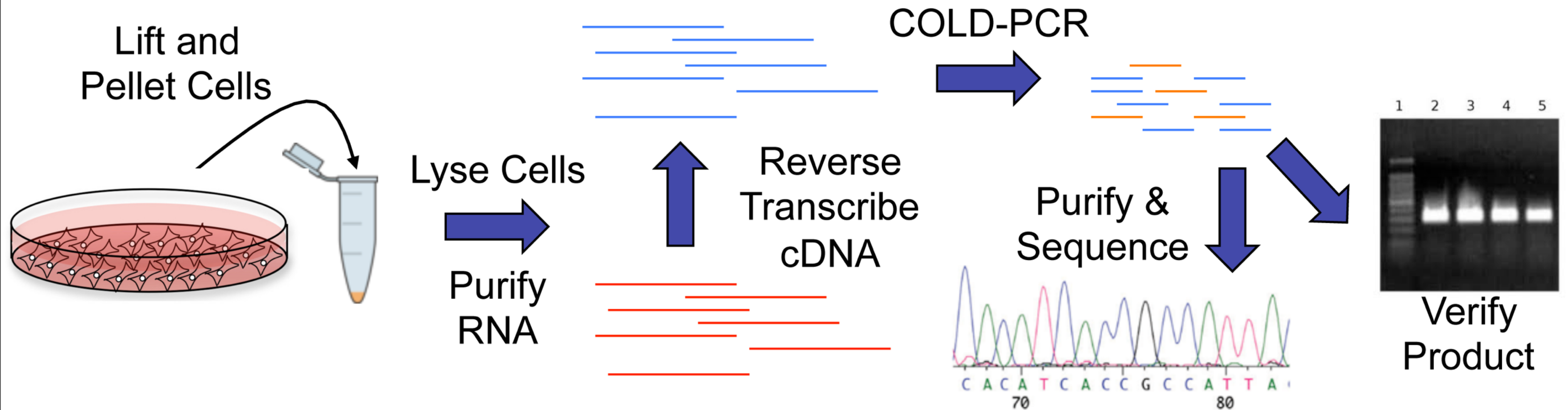

Positive Control:

Negative Control:

Experiment:

Today in the lab:

- Clean up your bench & grab a lab coat! Get cells from ice bucket up front -- get your own ice :-)
- RNA purification + RT set-up
- Atissa here @ 3:30pm
- Set-up PCR reactions -- thank Kim on the way out

Next time in the lab:

- Choose JC day & paper
- Analyze sequencing data
- Seed SKOV3 cells for phospho-WB
- Present Kirouac et al. paper -- I will switch my OH to Wednesday evening next week.