M2D2: Western Blot & System Conditions

03/10/16

1. Pre-lab discussion
2. Lyse cells
3. Measure total protein concentration
4. Western Blot Analysis: SDS-PAGE & Transfer to nitrocellulose
5. Investigate DNA repair sensor — pick your damage conditions (add to discussion page)

MOD2 Major Assignments

- Journal Club Presentation (10%)
  in class March 17th or April 7th

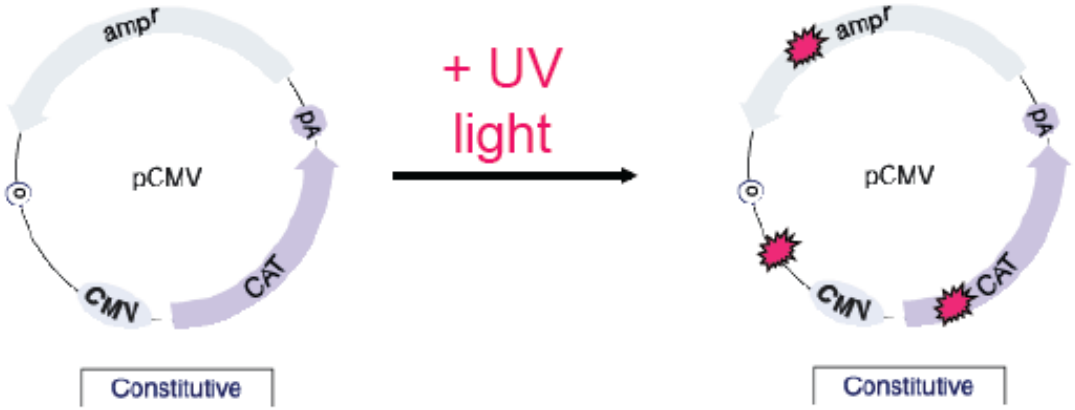
- System Engineering Research Article (25%)
  due at 5pm on Monday, April 18th

- M2D3 Homework:

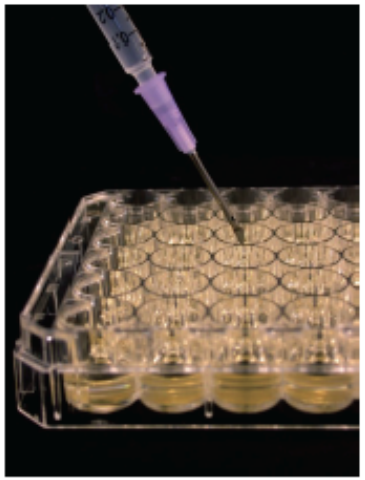
1) Schematic diagram (Figure, title and caption) of the NHEJ reporter plasmid with all features ***relevant*** to the NHEJ assay labeled.
2) Diagnostic digest calculation

Reactivation of UV damaged DNA by Host cell Reactivation (HCR)

Athas & GROSSMAN
Cancer Res. 1991



Transient transfection peripheral blood lymphocytes



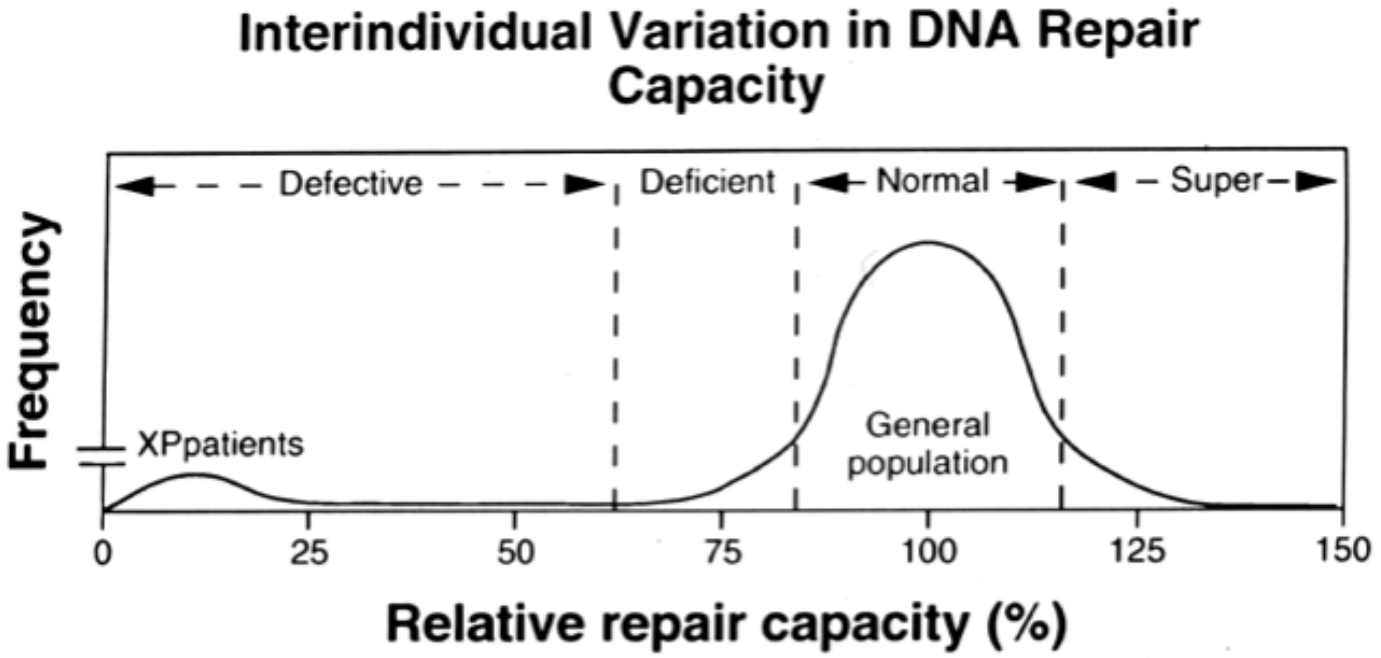
repair=CAT enzyme functional
no repair= No CAT enzyme

Time to repair

CAT Assay

Slide from M2D1 Lecture — Prof. Samson

assay found variability in human population in regards to DNA repair



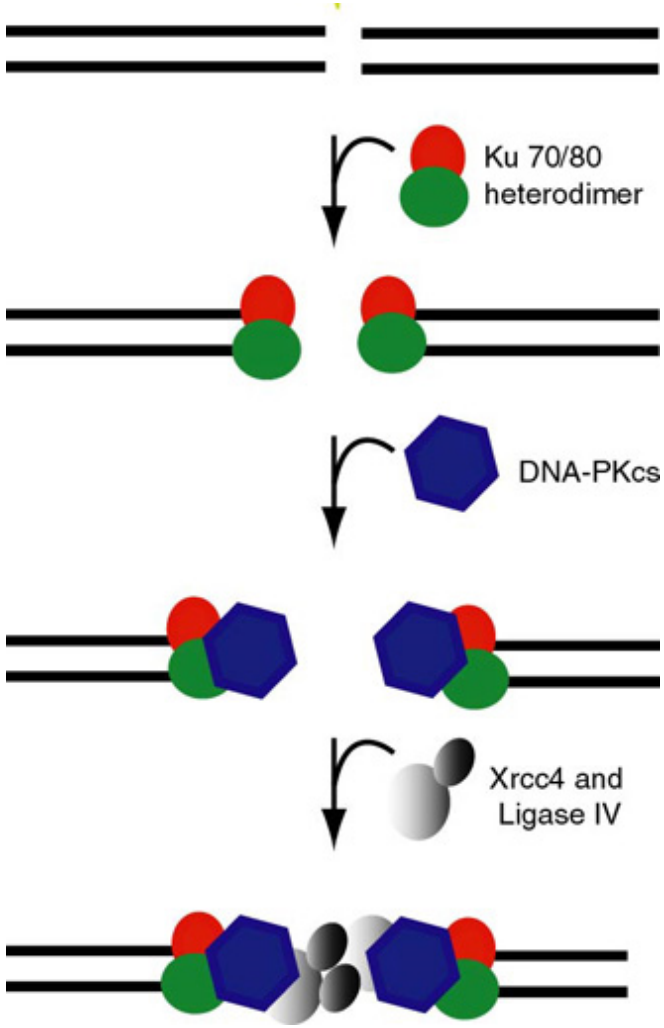
Adapted from **GROSSMAN and Wei (1995)** Clinical Chem 41: 1854-1863

XP frequency = ~1:250,000 giving a theoretical maximum of ~28,000 cases worldwide with 2,000-fold increased risk

Even if just 1% of the population is relatively repair deficient, could have tens of millions with several-fold increased risk

Slide from M2D1 Lecture — Prof. Samson

Non-Homologous End Joining (NHEJ)



Ku70
Ku80

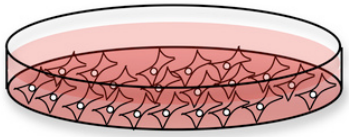
DNA-PKcs

Xrcc4
Ligase IV

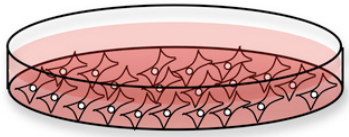
Slide from M2D1 Lecture — Prof. Samson

Module 2 Experimental Goal:

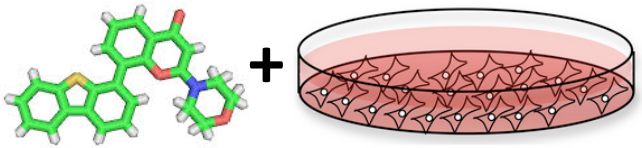
How efficiently does DNA repair via NHEJ act on DNA damage with different topologies?



"Normal cells"
M059K

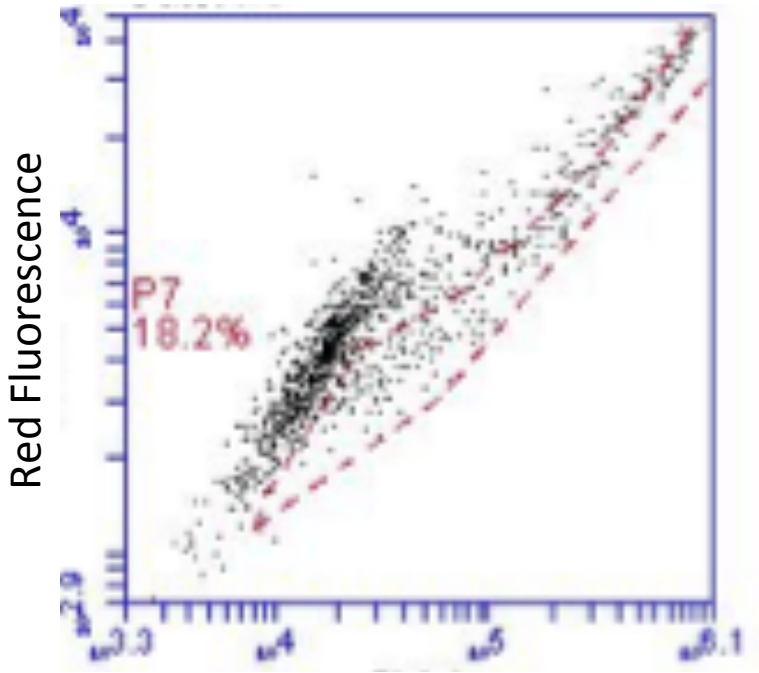


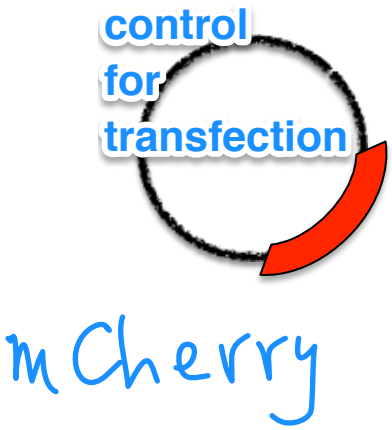
"DNA repair-deficient cells"
M059J

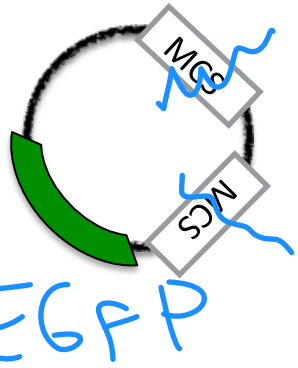


"Normal cells +
inhibitor of DNA repair"

Repair by NHEJ

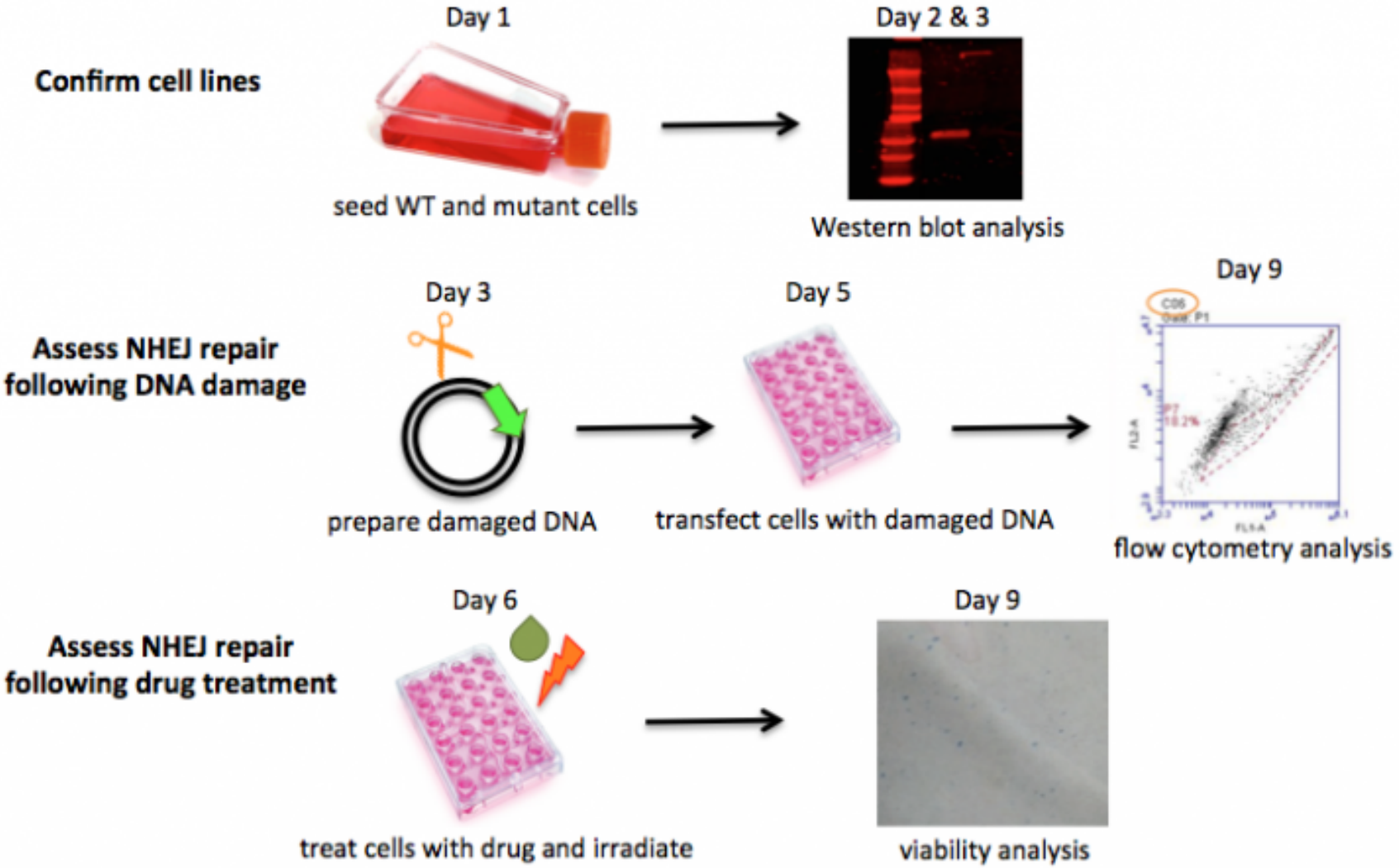




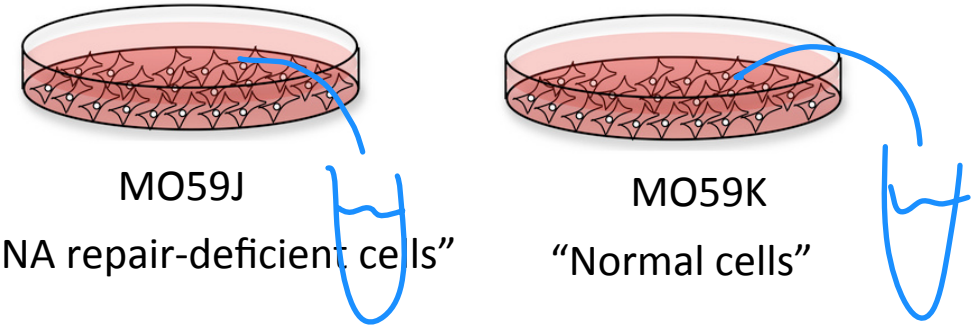


RE cut plasmid: to control the cut type
repair=green
no repair=no green

Mod 2 experimental overview



Validation of the experimental system:



MO59J

"DNA repair-deficient cells"

MO59K

"Normal cells"

breaks all membranes

Mammalian Lysis Buffer, RIPA:

-1% NP40 ; 0.1% SDS;

0.5% sodium deoxycholate

strong detergents

-protease inhibitors

stop protein degrdation

-Tris-HCl ph7.4: NaCl

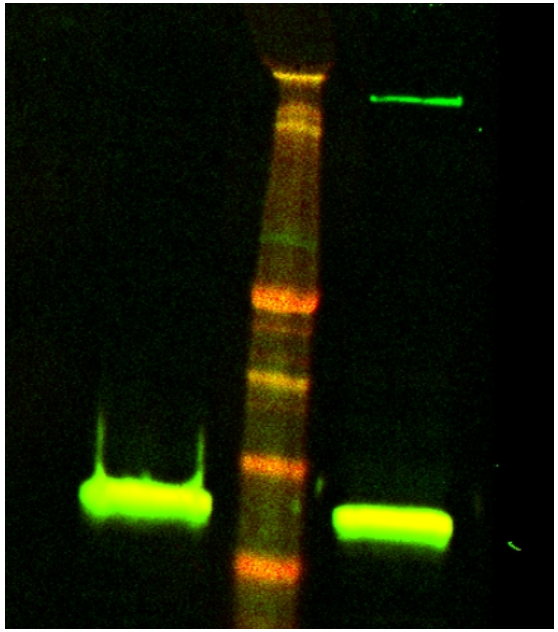
physiological ph and salt concentration

Cell lysate protein concentration measured using Precision Red Protein Assay

LI-COR Western blot

secondary dye imaged in far red

MO59J MO59K

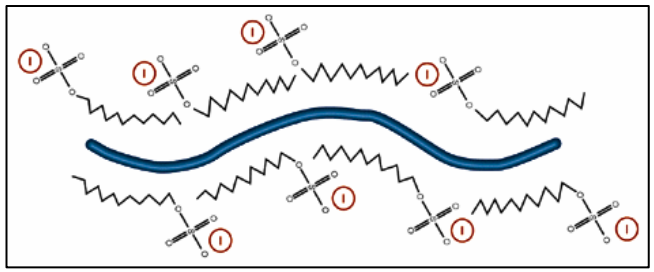


Western blot probed with:

anti-DNApk

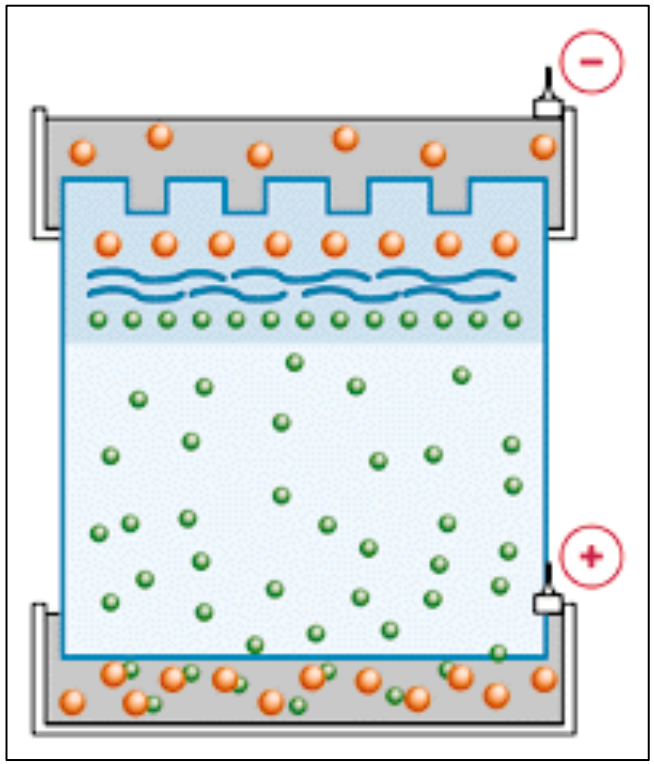
anti-tubulin

Western Blot Analysis (Step 1): SDS-PAGE



- Laemmli sample buffer / loading dye:

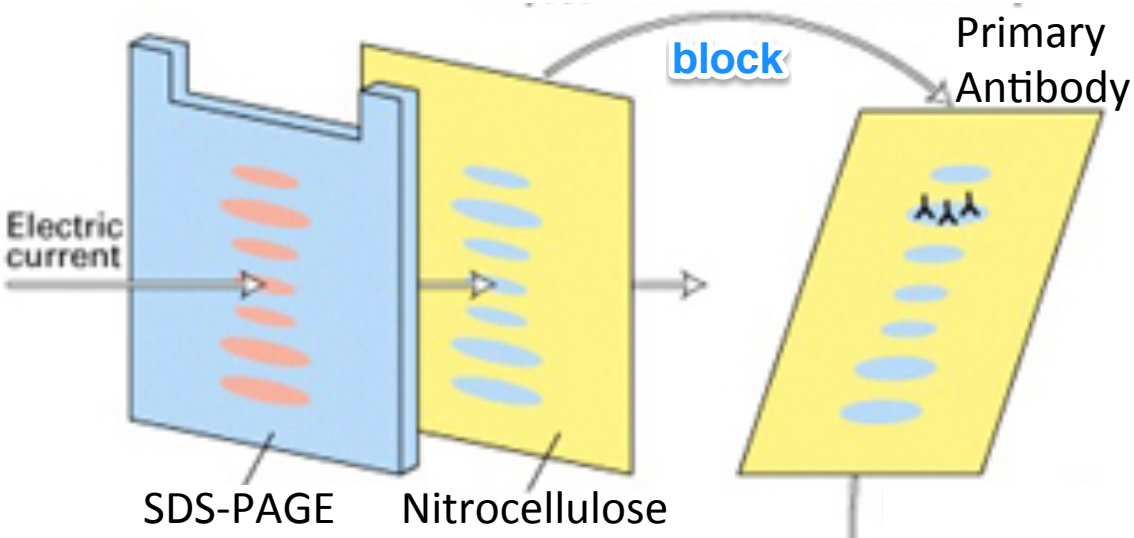
  SDS, BME, bromophenol blue



- boiling denatures higher-order structures
- TGS buffer
  - + Tris-HCl
  - + SDS/protein
  - + glycine

Hi Mark: prestained ladder for large MW proteins

Western blot Analysis (Step 2): Transfer and Immunoblotting



block

Primary Antibody

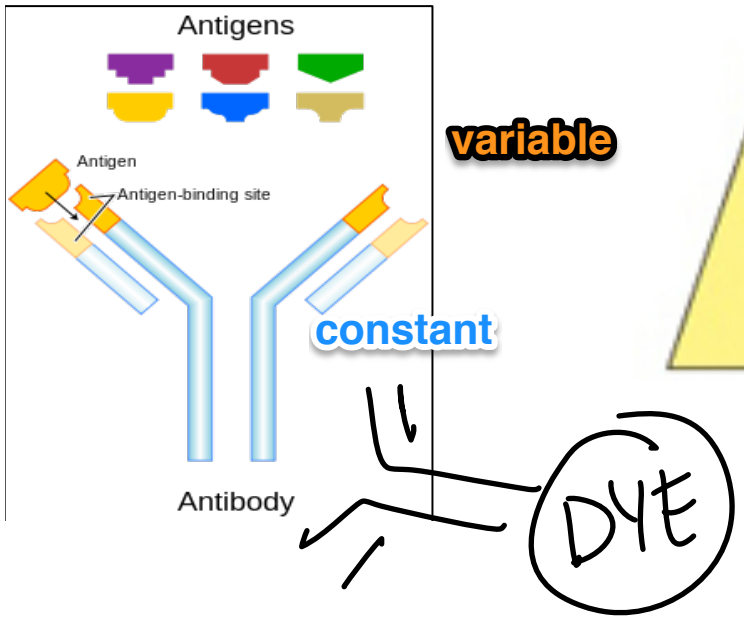
Dna dependent protein kinase
mouse anti-human

tubulin (loading control)
rabbit anti-human

Secondary Antibody

goat anti mouse 800 (pseudo green)

donkey anti rabbit 680 (pseudo red)

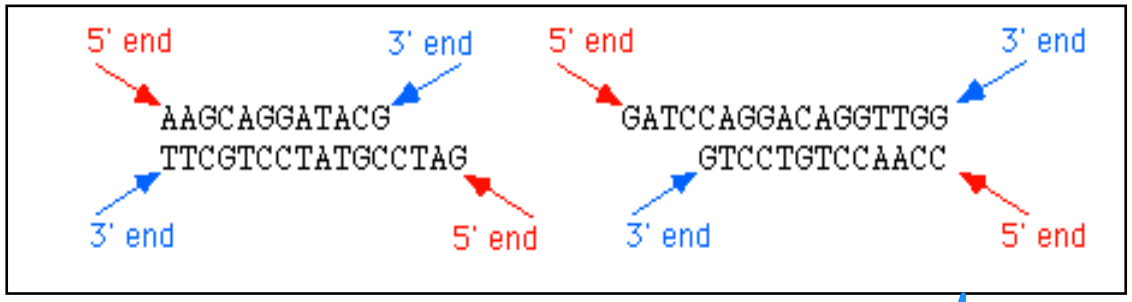


variable

constant

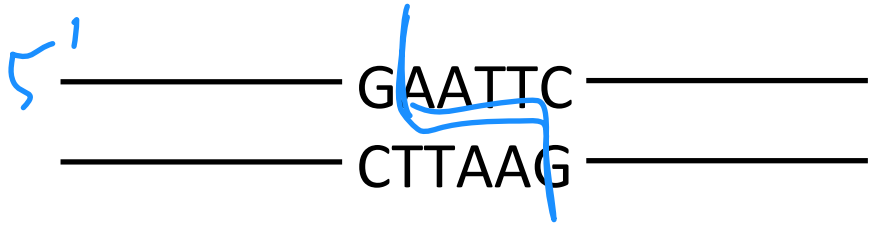
Restriction Enzymes digestion = DNA damage

5’ Overhang



3’ overhang
ex. PstI

5’ overhang
ex. EcoR1



Blunt
ex. PmeI

M2 Model of DNA damage:

Potential damage types:

blunt ends

compatible overhangs

incompatible overhangs

different color–
diff sequence

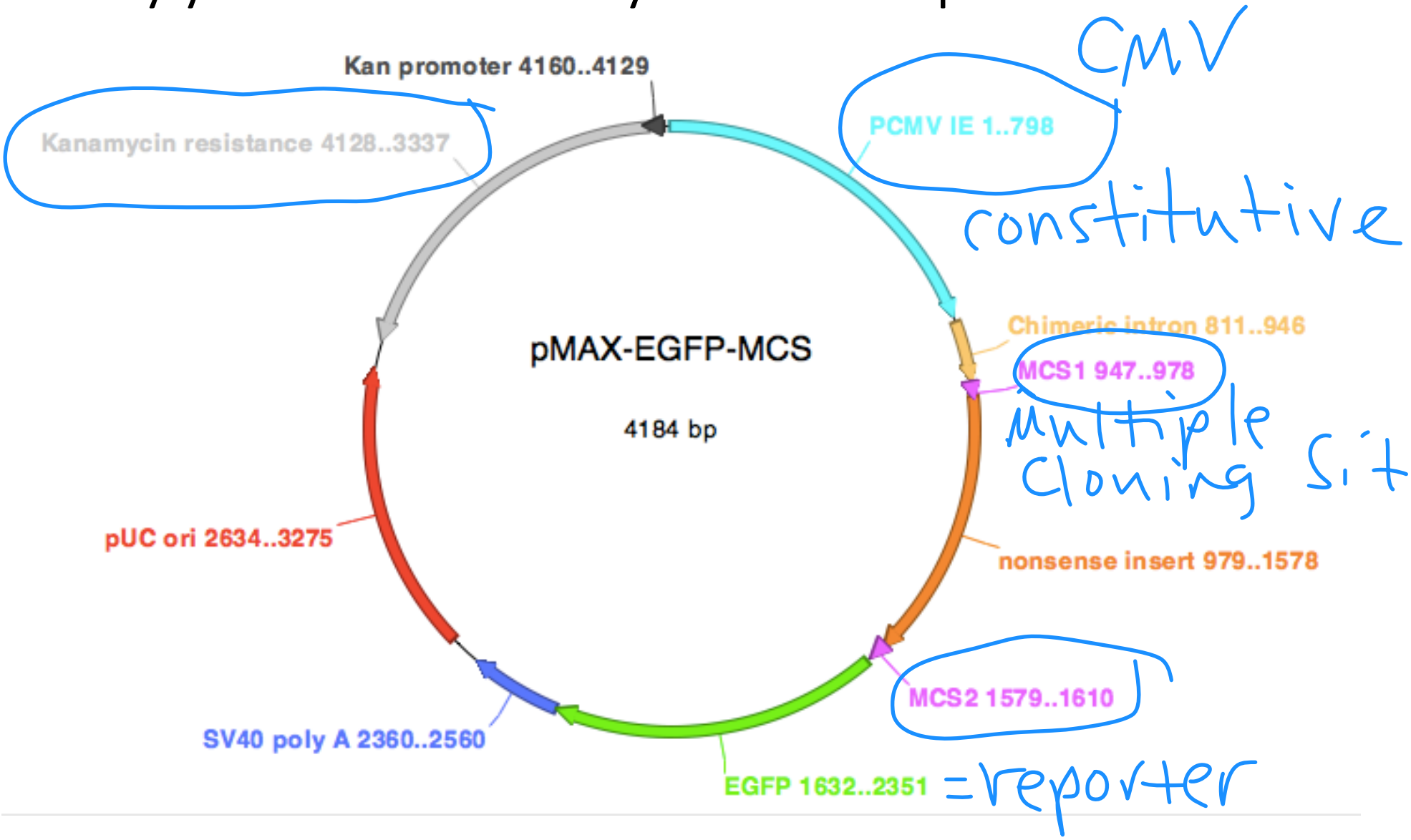
Hypothesis for NHEJ Repair capacity:

① easiest

② easier

③ hardest

Today you will familiarize yourself with pMAX-EGFP-MCS



Today in lab:

- Lyse cells on ice, keep lysate cold!
- Measure total protein concentration with Precision Red
- Load samples on SDS-PAGE
- Transfer protein to nitrocellulose membrane
- Familiarize yourself with the NHEJ reporter
- Add you DNA damage choice to wiki discussion page