

M2D2: Begin Western blot protein analysis and choose system conditions

03/11/2016

Key assignments of M2

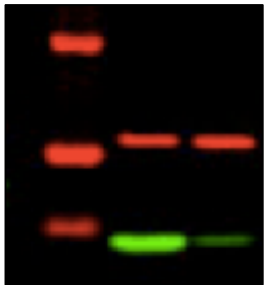


- Journal club presentation
 - 10%
 - individual
 - in class at 1pm on Friday, March 18 or April 8



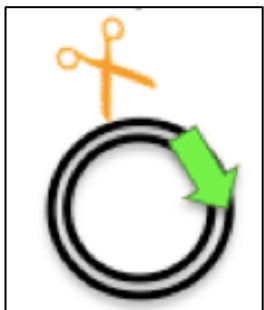
- Research article
 - 25%
 - individual
 - due 5pm on Monday, April 18
 - no draft/revision this time around

In lab today



1. Verify cell lines by Western blot protein analysis

- Lyse M059K and M059J cells
- Measure protein concentration
- Separate proteins by SDS-PAGE
- Transfer proteins onto nitrocellulose membrane



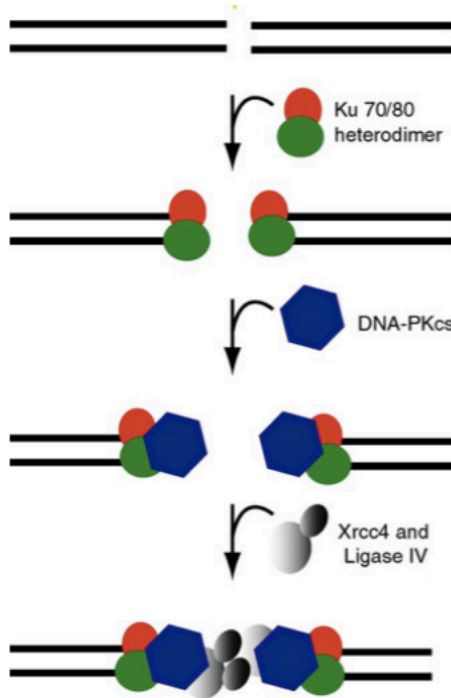
2. Pick DNA double strand break type

- Study DNA repair reporter of NHEJ
- Pick restriction enzymes' cut ends

1. Verify M059J is missing DNA-PKcs

DNA-dependent protein kinase catalytic site

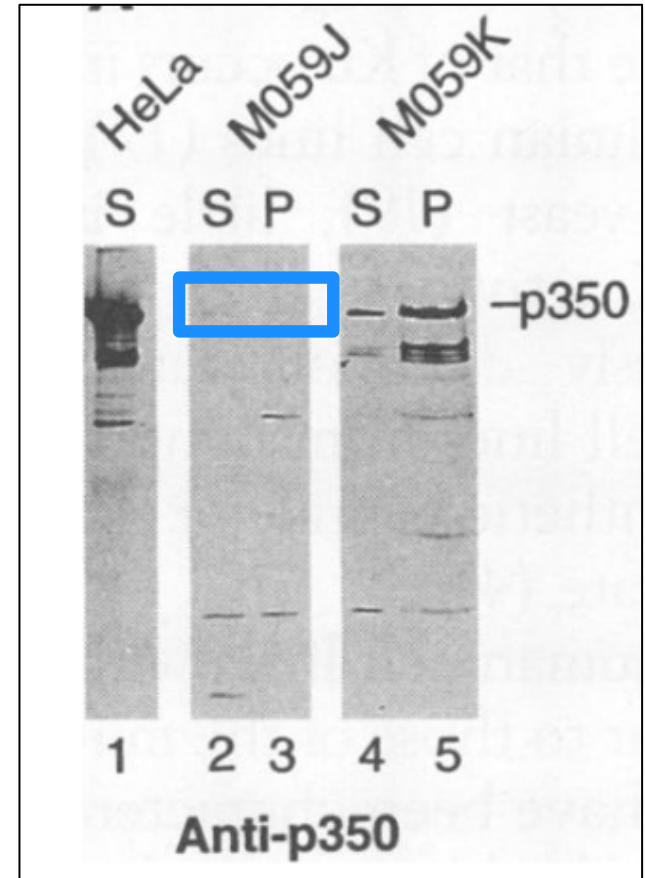
Non-Homologous End Joining (NHEJ)



Ku70
Ku80

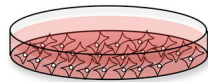
DNA-PKcs

Xrcc4
Ligase IV

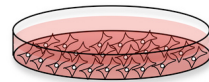


1. Verify cell lines by Western blot protein analysis

– Lyse M059K and M059J cells



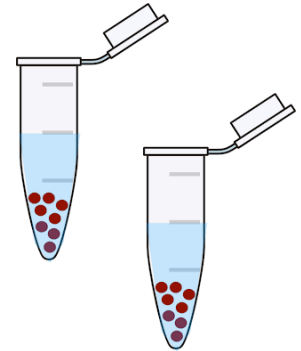
“normal cells”
M059K



“DNA repair-deficient cells”
M059J



proteins in
supernatant



RIPA buffer for mammalian cell lysis:

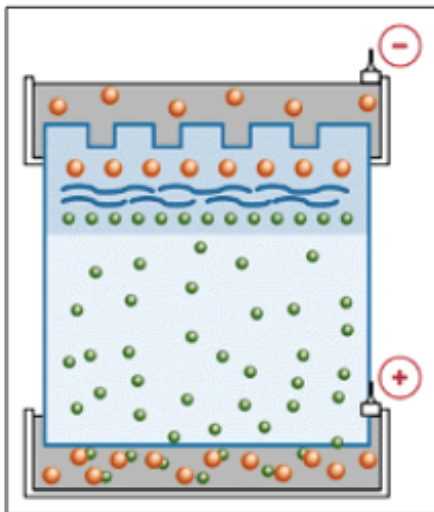
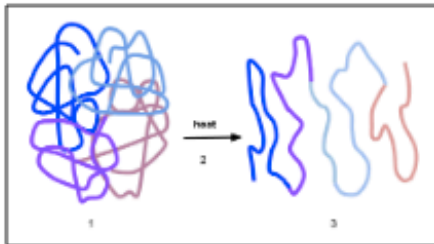
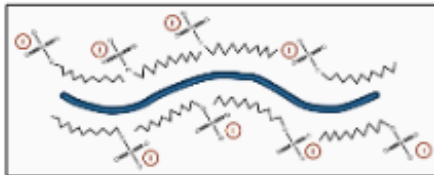
- **detergents:**
 - 1% NP-40 (nonyl phenoxyethoxyethanol)
 - 0.1% SDS (sodium dodecyl sulfate)
 - 0.5% sodium deoxycholate
- protease inhibitors **stop/stall protein degradation**
- Tris-HCl, pH 7.4 + NaCl **physiological levels of pH and salts**

1. Verify cell lines by Western blot protein analysis

SDS-PAGE separates proteins by size

sodium dodecyl sulfate – polyacrylamide gel electrophoresis

carcinogenic



- Laemmli sample buffer / loading dye:

- + SDS: **detergent denatures proteins, coats proteins with negative charges**


- + β -mercaptoethanol **reduces disulfide bonds**

- + bromophenol blue **to follow front of migration**

- + **glycerol**

- boiling denatures higher-order structures

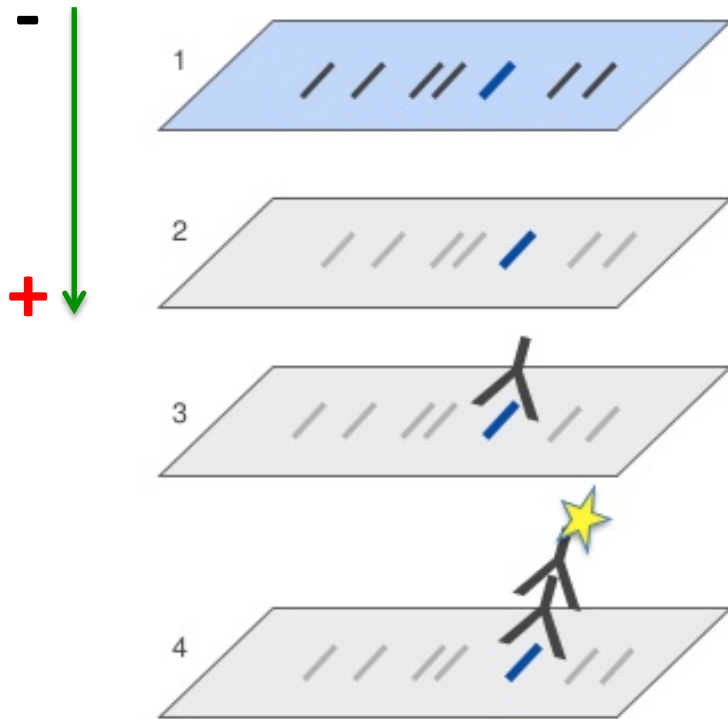
- TGS buffer **: sandwiched proteins form tight bands**

- + Tris-HCl 

- + SDS 

- + glycine 

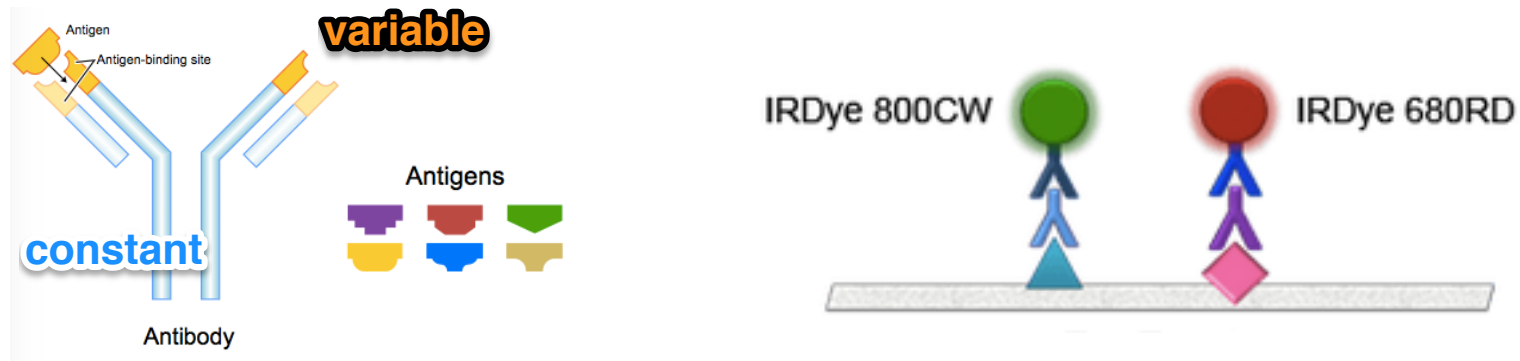
Western blot workflow











1. Protein separation by SDS-PAGE
 - HiMark stained ladder **bands: 31 - 460 kDa**
2. Protein transfer to nitrocellulose membrane
 - **high affinity for proteins**
 - **immobilizes proteins**
3. (Blocking and) probing with primary antibodies specific to
 - **DNA-PKcs p350 subunit**
 - **alpha-tubulin**
4. Probing with labeled secondary antibodies specific to primary antibodies
5. Image fluorescence signal

dual color, infrared fluorescence

Suite of antibodies for *LI-COR* Western blot



protein of interest	 DNA-PKcs	 tubulin
primary antibody	 mouse anti-human anti-DNA-PK	 rabbit anti-human anti-tubulin
secondary antibody	 goat anti-mouse	 donkey anti-rabbit
fluorescent dye IR wavelength	800 nm	680 nm
pseudo-color	 green	 red
molecular weight	~ 465 kDa	~ 50 kDa

top of membrane

bottom of membrane

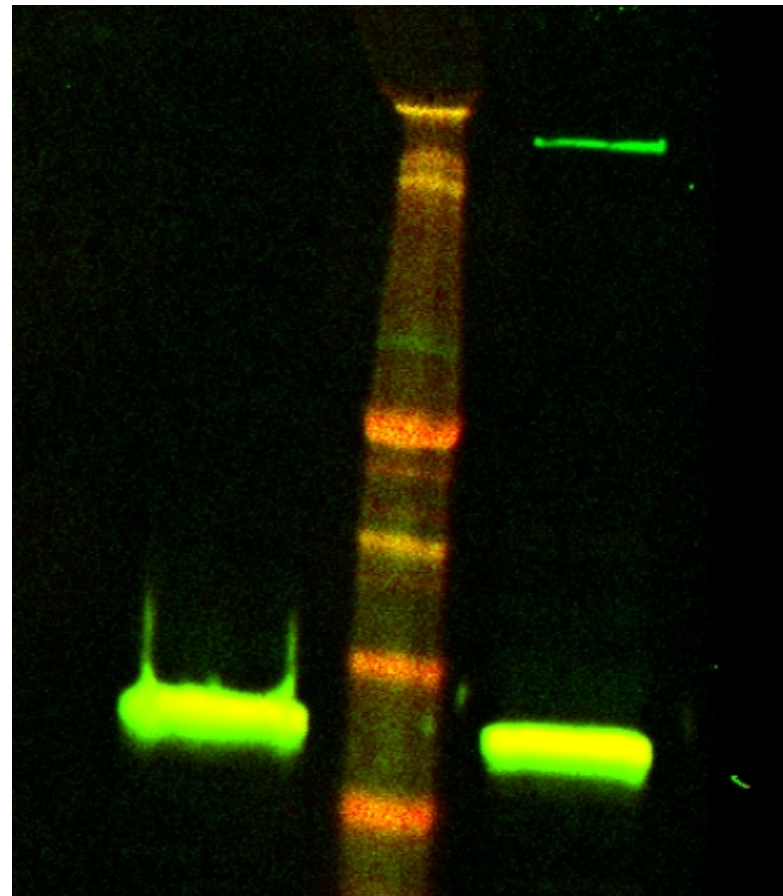
1. Verify M059J is missing DNA-PKcs by *LI-COR* Western blot

M2D3

M059 J

M059 K

MW



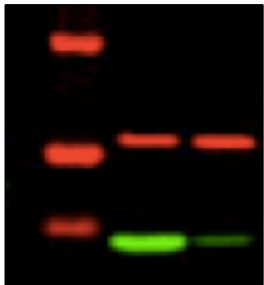
460 kDa p350 subunit

117 kDa

55 kDa

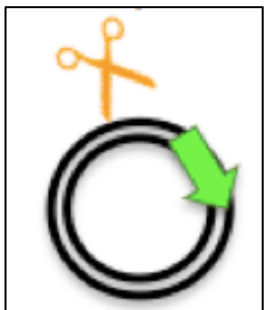
also in red channel !

In lab today



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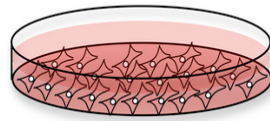
2. Pick DNA double strand break type

- Study DNA repair reporter of NHEJ
- Pick restriction enzymes' cut ends

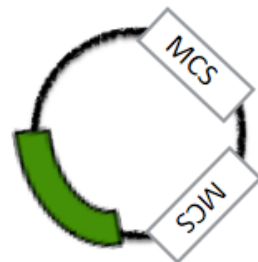
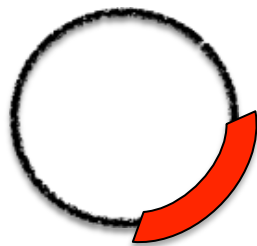
MCS: multiple cloning site, containing recognition sequences for many restriction endonucleases

M2: Quantify NHEJ in fibroblasts, DNA repair capacity by host cell reactivation

- “normal cells” **M059K** or
“DNA repair-deficient cells” **M059J**
(= no p350 subunit)

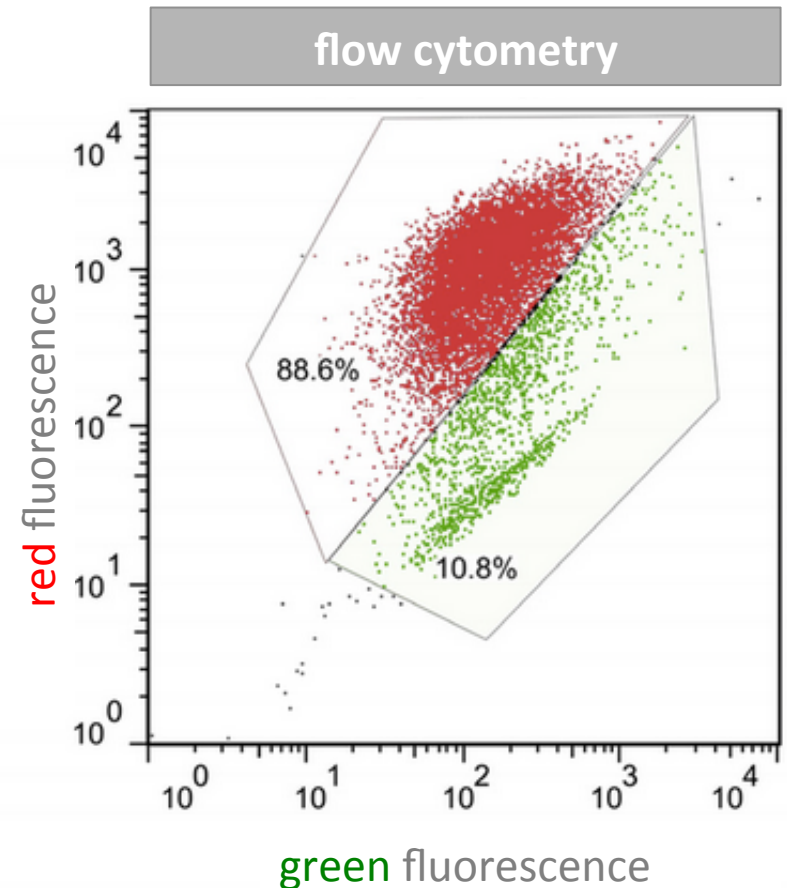


- transfect with DNA repair reporter
cuts with blunt vs. overhang ends

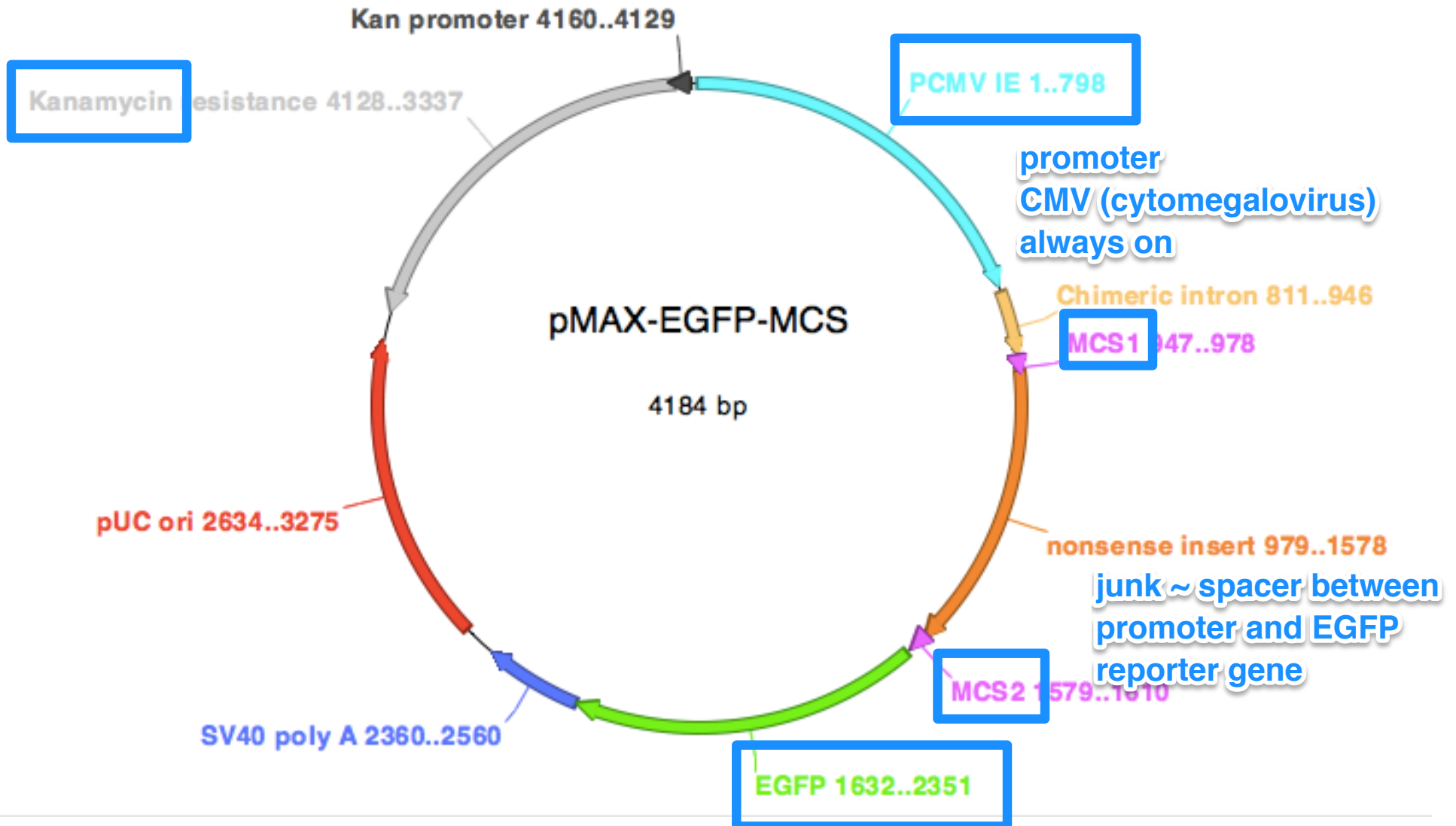


mCherry ~ RFP
control for
transfection

repair => green fluorescence




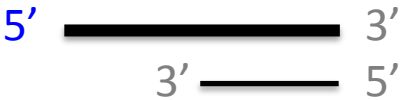

2. Pick DNA double strand break type
 - Study DNA repair reporter of NHEJ



2. Pick DNA double strand break type




– Pick restriction enzymes' cut ends

in MCS1
and MCS2

ends	example
<p>blunt</p> 	<p>5'... G T T T A A A C ... 3' 3'... C A A A T T T G ... 5'</p> <p>PmeI</p>
<p>5' overhang</p> 	<p>5'... G A A T T C ... 3' 3'... C T T A A G ... 5'</p> <p>EcoRI</p>
<p>3' overhang</p> 	<p>5'... C T G C A G ... 3' 3'... G A C G T C ... 5'</p> <p>PstI</p>

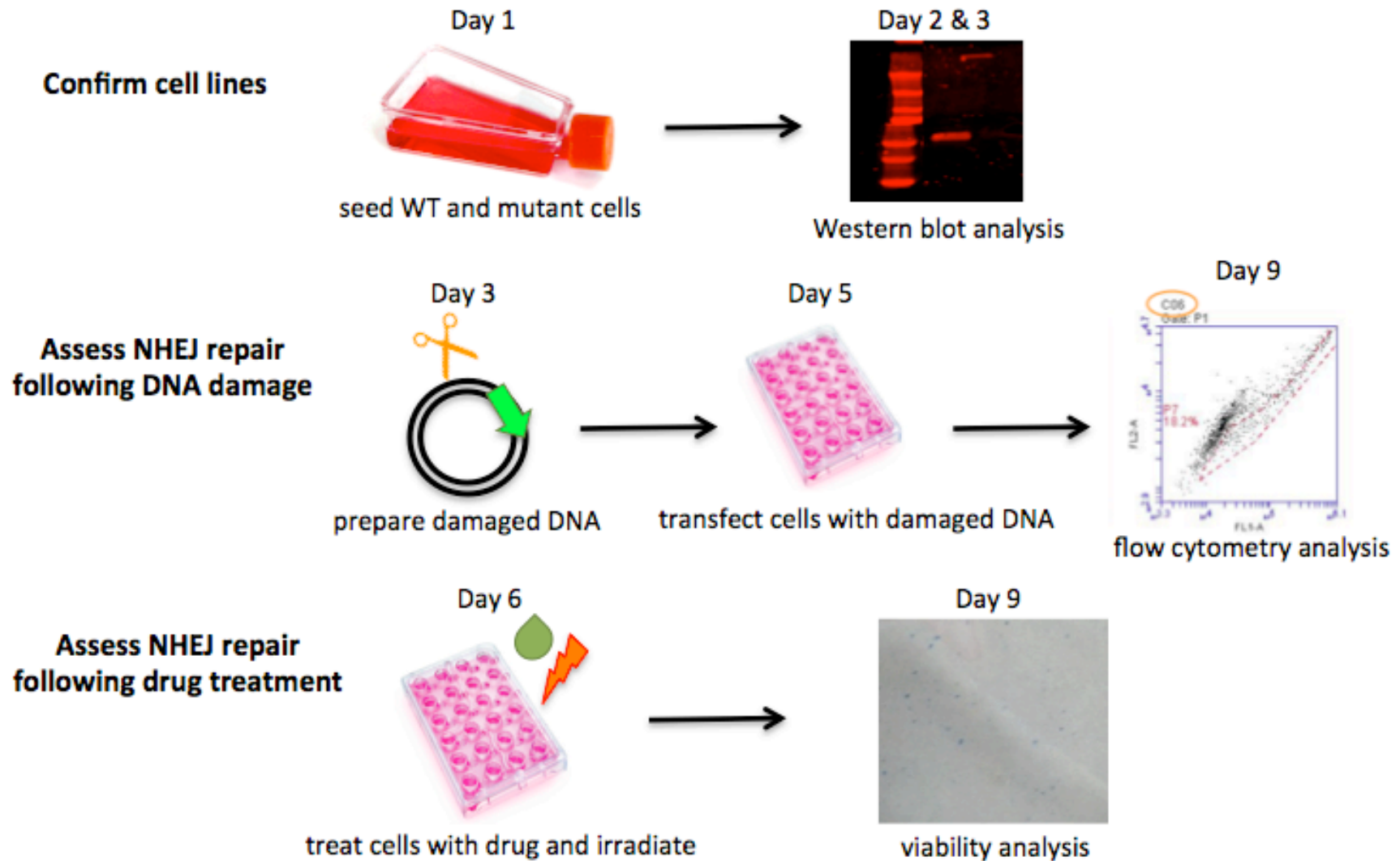
How efficient is NHEJ at repairing different types of double-stranded breaks (DSBs)?

vote!

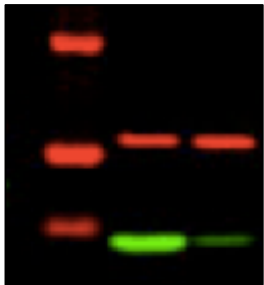
damage type	NHEJ repair capacity rank
<p>blunt ends</p> 	<p>4 votes for most efficient NHEJ repair</p>
<p>compatible overhangs</p> 	<p>6</p>
<p>incompatible overhangs</p> 	

* and sequence mismatch

M2 experimental overview



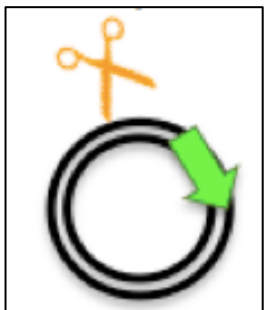
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Faculty will add blocking buffer tonight



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... and for M2D3: digest calculations