M2D3:
Complete Western blot and prepare damaged DNA

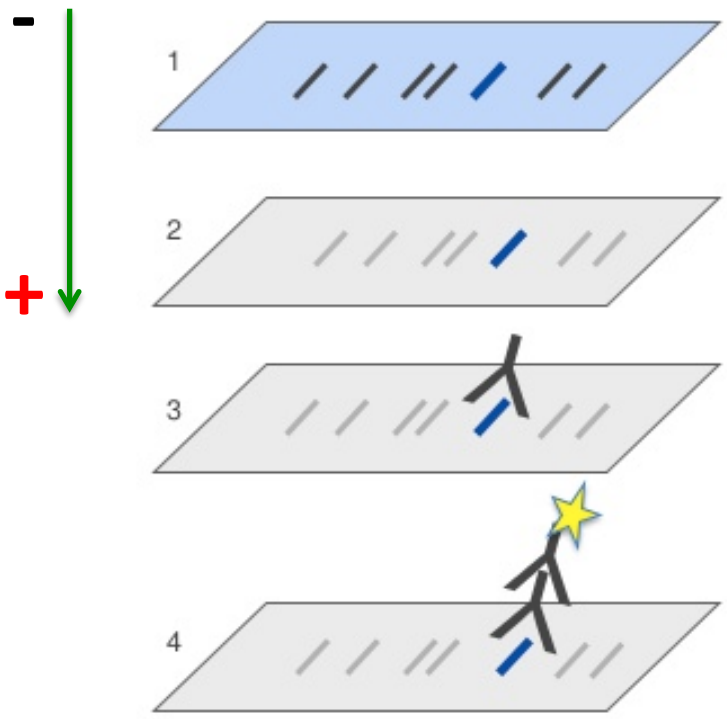
03/16/2016

Upcoming assignments

- M1 mini-presentation
  - email by 10pm tonight
  - bioeng20.109@gmail.com

- Journal club presentation
  - in 16-336 on Friday 03/18
  - post slides on Stellar before 1pm
  - choice of presentation order

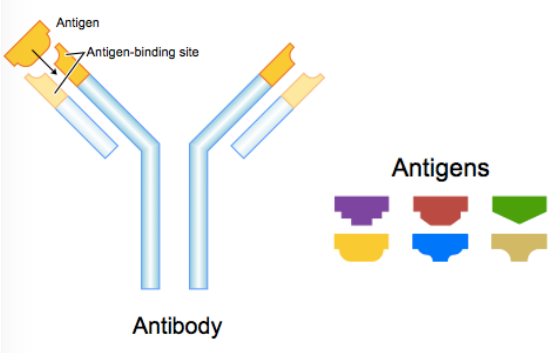
Western blot workflow:

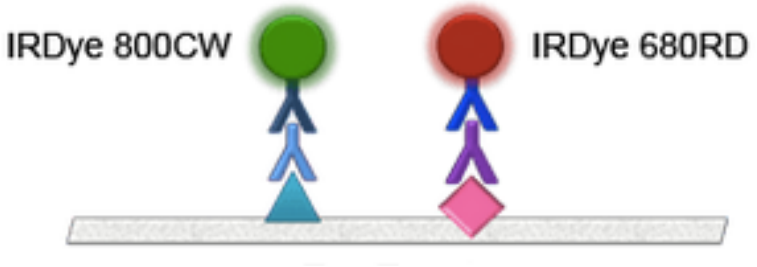


1. Protein separation by SDS-PAGE
2. Protein transfer to nitrocellulose membrane
3. Block membrane
4. Probe with primary antibodies specific to
   - DNA-PKcs
   - tubulin
5. Wash with TBS-T
   - **Tris-buffered saline with 0.1% Tween (detergent)**
   - to **destroy nonspecific interactions**
   - and to **wash away excess primary antibodies**
6. Probe with labeled secondary antibodies specific to primary antibodies
7. Wash
8. Image *LI-COR* fluorescence signal

from GE Healthcare

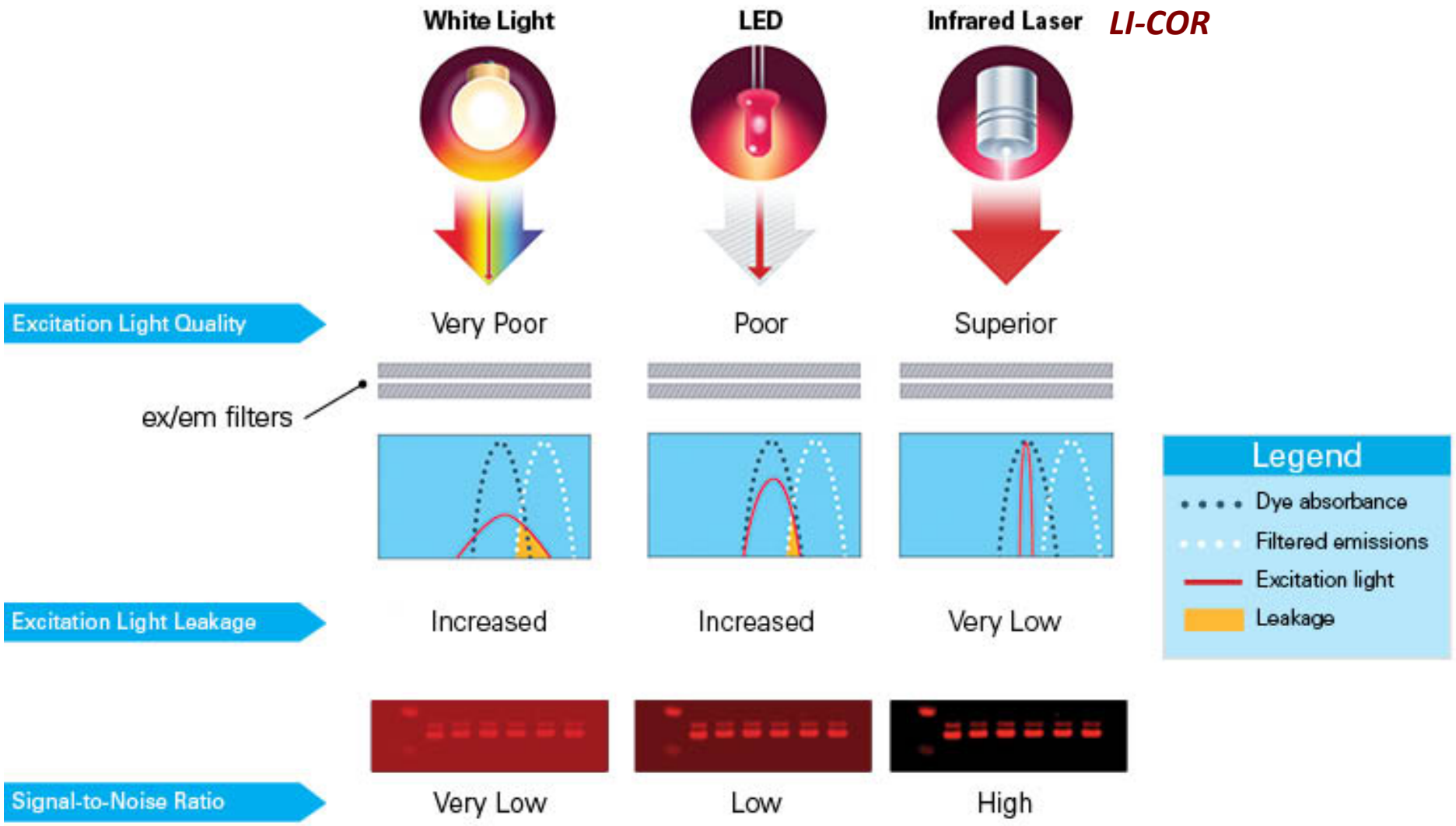
Suite of antibodies for *LI-COR* Western blot





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

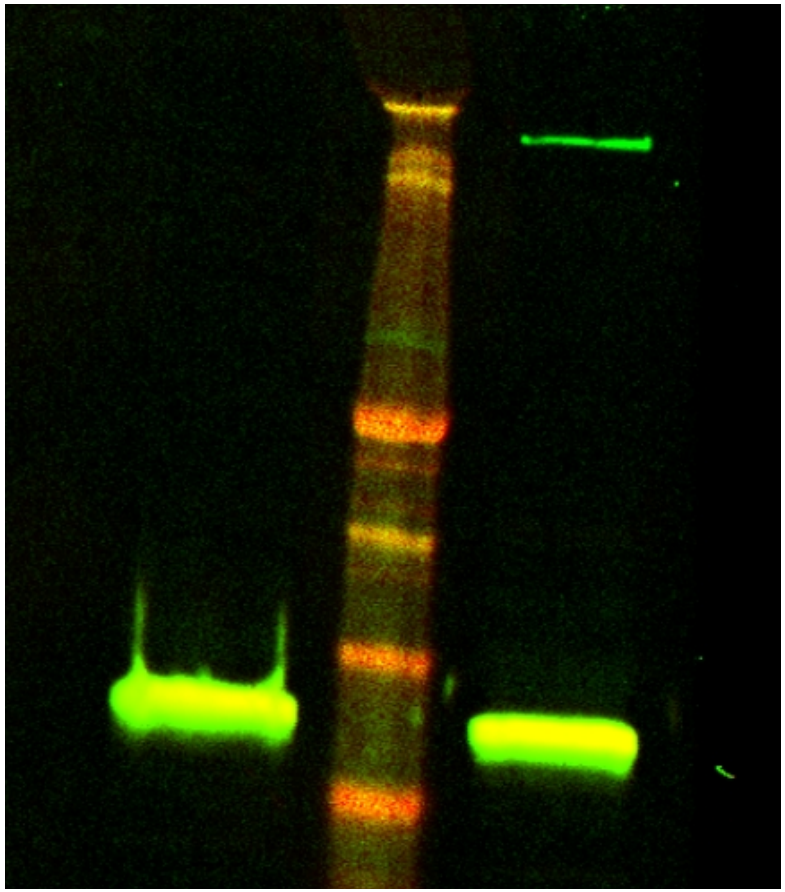
Near-IR: low auto-fluorescence background



licor.com, odyssey/sensitivity

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

M059J M059K MW



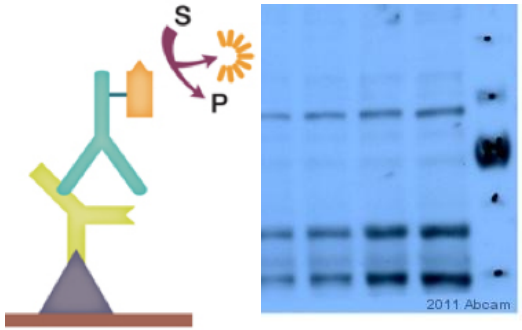
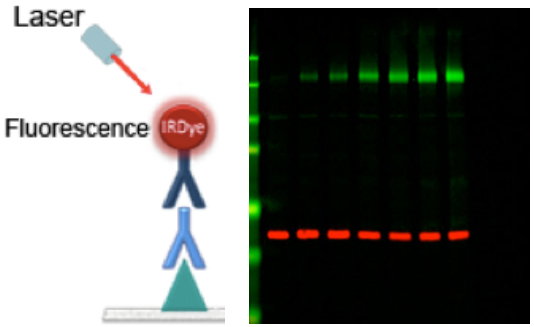
460 kDa

117 kDa

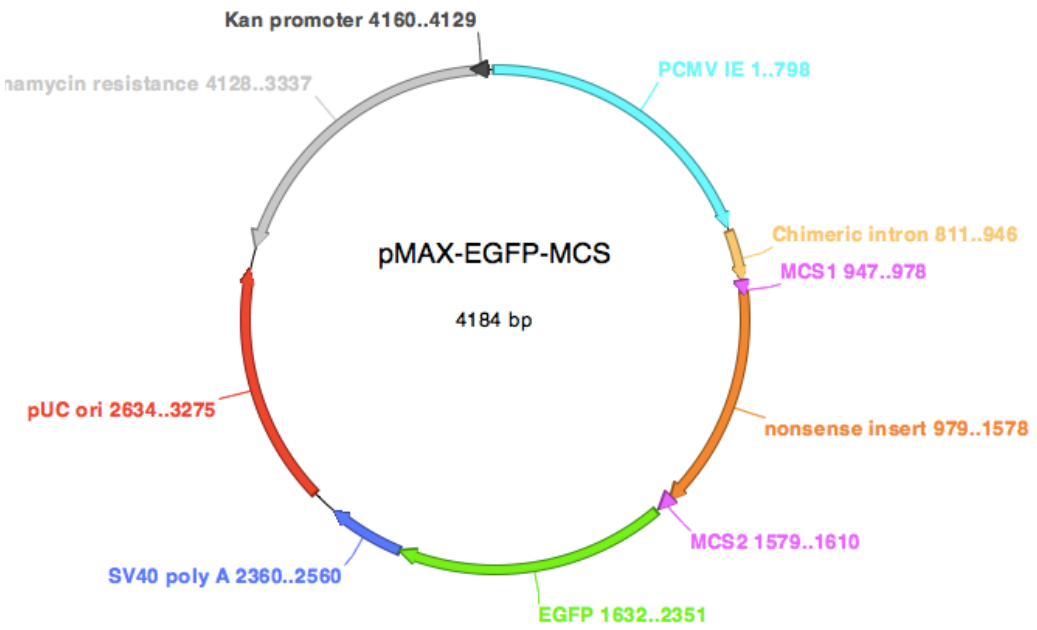
55 kDa

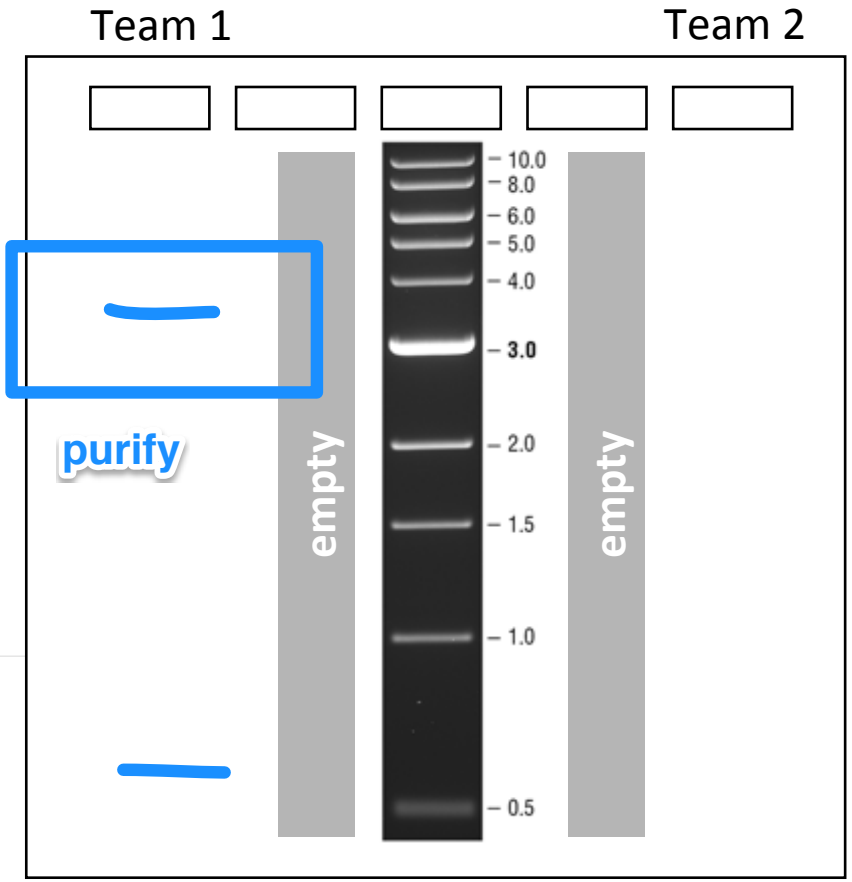
tubulin in “red” channel too

Western blot detection / visualization

Colorimetric	Chemiluminescent	Fluorescent
		
Upon incubation with a substrate that reacts with reporter (*e.g.* peroxidase), dye rendered insoluble and colored precipitates on membrane.	Incubation substrate luminesces when exposed to reporter on secondary antibody.	The fluorescently labeled probe is excited by light and the fluorescence emission is detected by a photosensor such as a CCD camera.
Pro: inexpensive, easy, no equipment required	**Pro**: sensitive, fast, film developer is common	**Pro**: sensitive, stable, able to multiplex
Con: medium sensitivity	**Con**: requires trial and error, time-dependent snapshot	**Con**: expensive

Preparation of damaged pMAX-EGFP-MCS:





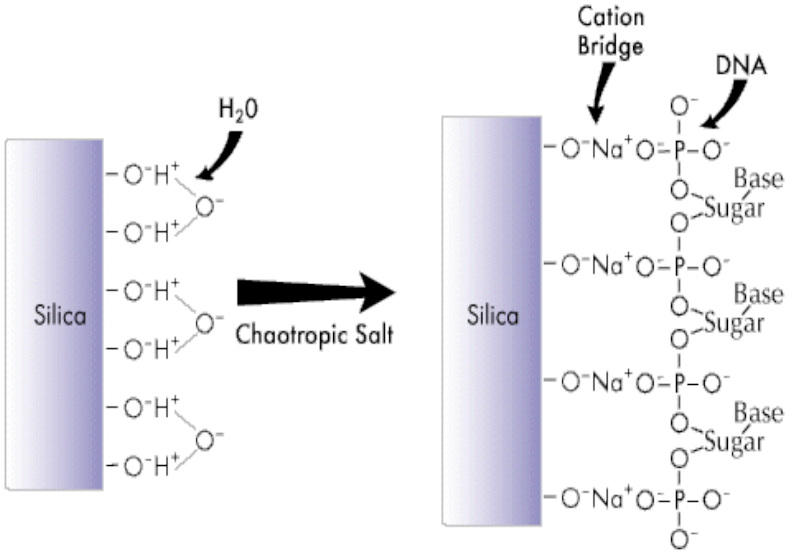
Restriction enzyme choices (buffer):

- *PmeI* CutSmart
- *BglII + EcoRI* 3.1
- *BglII + PstI-HF* 3.1

Add in this order: water + buffer + DNA + enzyme

Gel purification - Bind DNA to column

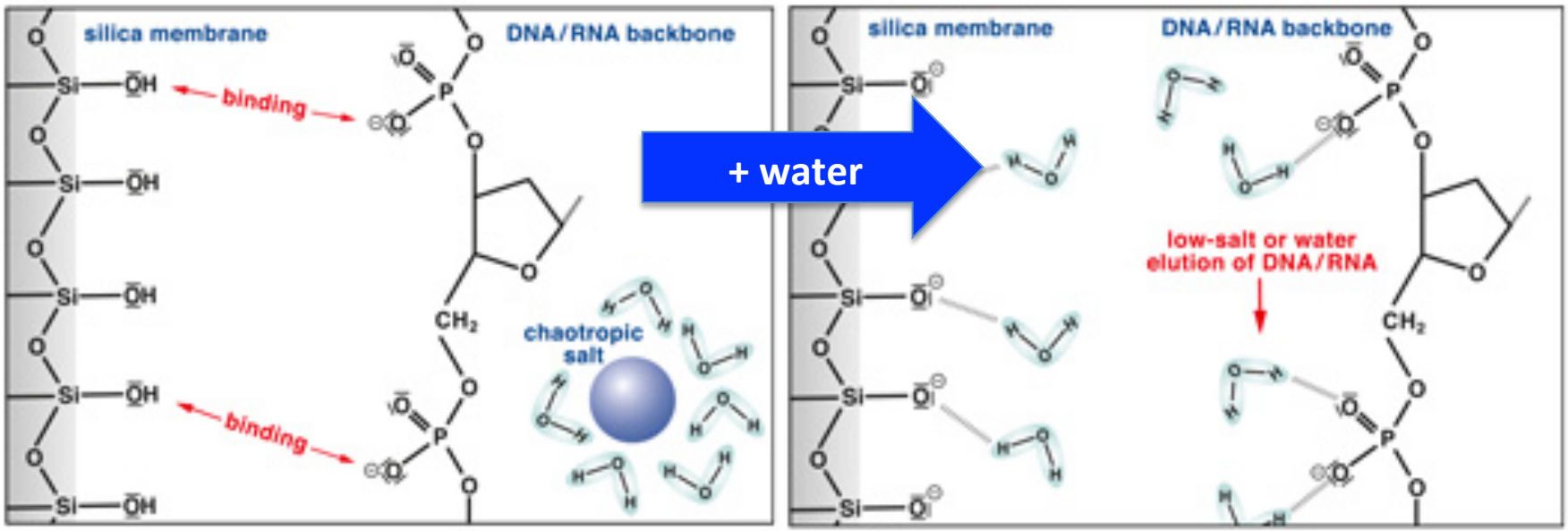
- Prepare with QG
  - dissolves agarose
  - chaotropic salt
  - pH indicator



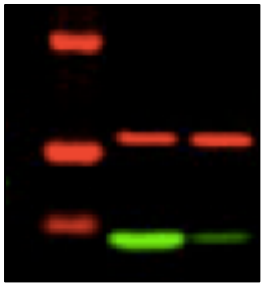
- Washes with PE
  - remove residual contaminants (eluent)
  - maintain DNA onto column

Gel purification - Elution with water

- Water (pH 8.0) competes DNA off of column



In lab today, split up:



- Set up restriction enzyme digest *(student 1)*
- Gel purify damaged DNA

- Complete immunoblotting *(student 2)*
  - save primary antibody solution
  - cover secondary antibodies with foil
- Image Western blot