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• check e-mail daily for announcements, etc..
  – I will post all questions received via e-mail, and the answers given, to the course mailing list. This way, everyone will have access to the information
    • 07528-W01@classes.uci.edu is the mailing list for the course
  – The registrar asserts that you have all been automatically subscribed - is this the case?
  – Anyone who does not have ready access to e-mail or the web should speak with me ASAP
  – If you object to your question being posted on the course mailing list, please indicate this clearly in the message..

• lectures will be posted on web page
  – as I have time.
General comments

- Final examination will not be cumulative, however, understanding of concepts and techniques from first part of course is required.

- Material covered will include Dr. LaMorte’s lectures on 3/1 and 3/6

- I will cover material that is not in either book. You are responsible for what I lecture about, not for any particular chapters in the books.

- Please be advised that Dr. La Morte's lectures will be held in the Beckman Laser Institute conference room
General comments (contd)

• Overall philosophy
  – Class is about understanding eukaryotic gene regulation, particularly how to study it.
  – Intended to be informative and cutting edge but also interesting and relevant, even fun.
  – I try to be available to students as much as possible
  – questions during lectures are welcome. Please stop me if something is unclear.

• Goal for 2nd half of the course
  – to familiarize you with current methods of studying gene regulation
  – to gain an understanding of which methods are best applied in a particular situation
  – to survey some important examples of gene regulatory systems and pathways

• Letters of recommendation
  – If you want more than form letter I need to know you as more than a student #
    • come to office hours
    • participate in class discussions
    • make your interest in the subject apparent
  – Good students get good letters
Lecture Outline 2/8/2001 - Introduction of cloned genes into eukaryotic cells, tissues and embryos

- methods to detect gene transfer
  - selection methods
    - antibiotics
  - reporter gene assays
    - types, advantages and disadvantages of each
Selection methods. How does one select for cells that have taken up the DNA of interest

• It is axiomatic among microbiologists that you can accomplish anything if you have an assay, and faster with a method of selection.
• There have been, and will continue to be, numerous publications describing improved methods of selection.
• Rule of thumb:
  – positive selection >>>> negative selection
• metabolic pathway selection (mostly of historical interest)
  – HGPRT and HAT selection
  – DHFR and other amplifiable markers
  – gpt and mycophenolic acid
• antibiotic resistance. All work both in bacterial and mammalian cells. Cost of all is comparable for use in mammalian cells.
  – G418
  – hygromycin
  – zeocin/phleomycin
  – puromycin
  – blasticidin S
• TK selection - widely used as a negative selection
  – gancyclovir
  – FIAU (fialuridine)
Positive selection - antibiotic resistance - G418

- source: aminoglycoside antibiotic related to gentamycin
- activity: broad action against prokaryotic and eukaryotic cells
  - inhibits protein synthesis by blocking initiation
- resistance - bacterial neo gene (neomycin phosphotransferase, encoded by Tn5 encodes resistance to kanamycin, neomycin, G418
  - but also cross protects against bleomycin and relatives.
Positive selection - antibiotic resistance - G418 (contd)

- Stability:
  - 6 months frozen

- Selection conditions:
  - E. coli: 5 µg/ml
  - Mammalian cells:
    - 300-1000 µg/ml. G418 requires careful optimization for cell types and lot to lot variations
    - Requires at least seven days to obtain resistant colonies, two weeks is more typical

- Use and availability:
  - Perhaps the most widely used selection in mammalian cells
  - Vectors very widely available
Positive selection - antibiotic resistance - hygromycin B

- source: aminoglycoside antibiotic from Streptomyces hygroscopicus.
- Activity: kills bacteria, fungi and higher eukaryotic cells by inhibiting protein synthesis
  - interferes with translocation causing misreading of mRNA
- resistance: conferred by the bacterial gene hph
  - no cross resistance with other selective antibiotics
- stability:
  - one year at 4 °C, 1 month at 37 °C
- selection conditions:
  - E. coli: 50 µg/ml
  - mammalian cell lines:
    - 50 - 1000 µg/ml (must be optimized)
    - 10 days- 3 weeks required to generate foci
- use and availability:
  - vectors containing hygromycin resistance gene are widely available
  - in use for many years
Positive selection - antibiotic resistance - zeocin/phleomycin

- source: glycopeptide antibiotic of the bleomycin family produced by a Streptomyces verticillus mutant
- activity: broad against bacteria, eukaryotic microorganisms, plant and animal cells in vivo
  - intercalating reagent -> DNA degradation
  - perturbs plasma membranes
- resistance: conferred by the bacterial ble gene
  - cross-resistance conferred by Tn5 neo gene.
  - Despite manufacturer’s claims to the contrary, this means that one must be careful when using this selection in E. coli since Tn5 is relatively common in laboratory strains.
- Stability:
  - 4 °C for several months, 37 °C for one week
- selection conditions:
  - E. coli - 5 µg/ml
  - mammalian cells
    - 5-50 µg/ml for Phleomycin, 25-1000 µg/ml for Zeocin (must be optimized)
    - 10 days- 3 weeks required to generate foci
- use and availability:
  - vectors containing zeocin resistance gene are now commercially available from InVitrogen
  - not much track record yet
Positive selection - antibiotic resistance - puromycin

- source: aminonucleoside antibiotic from Streptomyces alboniger
- activity: gram positive bacteria, animal and insect cells.
  - Gram negative bacteria and fungi are resistant due to low permeability
  - acts as an analog of 3’ terminal end of aminoacyl-tRNA of both prokaryotic and eukaryotic ribosomes causing premature chain termination
- resistance: bacterial pac gene encodes puromycin N-acetyl-transferase
  - no cross-resistance to other selective antibiotics
Positive selection - antibiotic resistance - puromycin (contd)

- Stability:
  - 4 °C for up to one year

- selection conditions:
  - E. coli: not active, therefore isn’t useful for selection
  - mammalian cells:
    - 3-50 µg/ml (must be optimized)
    - cells detach and die very rapidly - colonies in less than 7 days

- use and availability:
  - in use for many years
  - vectors not widely available
Positive selection - antibiotic resistance - blasticidin S

- source: peptidyl nucleoside antibiotic isolated from Streptomyces griseochromogenes
- activity: broad spectrum in prokaryotes and eukaryotes
  - inhibits peptide bond formation
- resistance: three resistance genes known
  - bsr - Bacillus, deaminase, commonly used in animal cells
  - BSD - Aspergillus, deaminase - commonly used in fungi and plant cells
  - bls - Streptomyces, acetyl transferase - not widely used
- Stability:
  - 4 ºC for up to one year
- selection conditions:
  - E. coli: 100 µg/ml
  - mammalian cells:
    - 3-50 µg/ml (must be optimized)
    - cell death occurs very rapidly allowing transformants to be selected in as little as 7 days
- use and availability:
  - been around for ~ 10 years
  - not widely used yet
Negative selection HSV-TK

- source: Herpes simplex virus encodes a thymidine kinase gene.
  - This was used to engineer resistance to HAT medium in older experiments. Cumbersome

- activity: presence of HSV-tk confers sensitivity to certain nucleoside analogs. This is widely used in current antiviral therapy, e.g. AIDS, Herpes, CMV, etc
  - converts these nucleoside analogs into toxic compounds
    - gancyclovir
    - FIAU (fialuridine)

- selection conditions:
  - FIAU - 0.2 µM - 0.2 mM is working concentration. needs considerable optimization
  - gancyclovir is quite variable and gives more non-specific toxicity than FIAU

- use and availability
  - very widely used as a negative selection in gene targeting experiments
  - touchy and difficult to optimize
Selection methods - summary

• Considerations
  – what is the goal of the experiment?
    • Are multiple, different constructs needed in each cell type?
      – E.g. constructing multiply marked chromosomes by homologous recombination
      – if not, most any selection will work
    • what is already working in the lab or surrounding labs?
  – Are there time constraints that must be addressed?
  – Short term vs long term goals
    • will the cell type require multiple rounds of selection?
    • Are there enough selective markers available or must they be recycled?

• positive vs negative selection
  – Positive selection virtually always works
    • methods are straightforward to optimize and very effective
    • negative selection must be carefully calibrated and optimized.
    • Even then, it frequently fails since the dose required to kill all of the undesirable cells also kills many desirable ones as well
Reporter genes and assays

- The goal is to detect the presence of the transferred gene
  - physical presence (e.g. mRNA)
  - biological activity
  - effect on other genes
- typical strategy is to engineer a DNA construct that reports the presence of a desirable feature, e.g., the activity of a promoter
  - two basic flavors exist
    - promoter constructs - promoter or promoter fragment is fused to a reporter marker
    - minimal promoters containing a response element fused to a reporter gene
- common reporter genes utilized - typically these are enzymes.
  - chloramphenicol acetyl transferase (CAT)
  - luciferase (luc)
  - β-galactosidase (β-gal)
  - β-glucuronidase (β-gus)
  - β-lactamase
  - secreted alkaline phosphatase (SEAP)
  - growth hormone (GH)
  - green fluorescent protein (GFP)
Reporter genes and assays (contd)

• important issues for reporter gene selection
  – What is the sensitivity required?
  – What is the cost of the enzymatic substrate vs the sensitivity required?
  – How many assays are required?
  – How convenient is it to do required transfection controls?
  – What is the equipment required?
  – What is the dynamic range of the assay?
    • What is the difference between the lowest and the highest activity detectable in the same reaction without dilution?
    • Very important for high-throughput assays
  – Is in vivo detection required?
  – What reagents are readily available vs when you need to do the assays?
Typical reporter constructs - 1

- promoter analysis vector
  - required features
    - readily detectable reporter gene
    - no eukaryotic regulatory sequences
      - promoters
      - enhancers
    - multiple cloning site
    - bacterial origin of replication
    - antibiotic resistance for bacterial selection
  - nice extras
    - eukaryotic selection
Typical reporter constructs - 2

- enhancer analysis vector
  - required features
    - readily detectable reporter gene
    - minimal promoter sequence, e.g., TATA box, transcription initiation site
      - SV40
      - Herpes thymidine kinase
      - heat shock
    - multiple cloning site upstream
    - bacterial origin of replication
    - antibiotic resistance for bacterial selection
  - nice extras
    - eukaryotic selection
Reporter genes 1 - CAT

- chloramphenicol acetyl transferase
- enzyme catalyzes the addition of acetyl groups from acetyl-CoA to $[^{14}\text{C}]-\text{chloramphenicol (CAP)}$
- methods
  - simple biochemical reaction - incubate cell extracts with substrates, extract into ethyl acetate, dry, resuspend and spot on TLC plate
  - TLC assay - separation of acetylated products by thin layer chromatography and detection by autoradiography
    - modest linearity - not particularly quantitative
    - sensitivity is $\sim10x$ lower than LSC assay
    - VERY tedious for multiple samples
Reporter genes 1 - CAT

- Methods (contd)
  - LSC assay - substitute butyryl-CoA for acetyl-CoA. Butyryl-CAP is soluble in xylene whereas CAP is not. Partition reaction between xylene and aqueous phase. Count organic phase.
    - linear for nearly three orders of magnitude
    - sensitivity $3 \times 10^{-4}$ units of CAT (1 unit transfers 1 nmol of acetate to CAP in one minute at $37^\circ C$.
    - sensitivity is $\sim 20$ pmol
Reporter genes 1 - CAT (contd)

- Methods (contd)
  - ELISA - antibody-based assay (CAT doesn’t need to be active)
    - lyse cells, bind extract to micotiter plate wells, detect peroxidase activity
    - 10 pg/well sensitivity \(\approx 1\) pmol
    - ELISA > LSC > TLC assay
  - sensitivity depends on the nature of the peroxidase substrate used.
Reporter genes 1 - CAT (contd)

- advantages
  - little or no equipment required TLC tank or scintillation counter (plate reader for ELISA)
  - widely used in literature
- disadvantages
  - sensitivity is modest
  - dynamic range is not so great (max 3 decades for ELISA, less for radioactive)
  - radioactive assay - $^{14}$C is most expensive isotope to dispose of
    - very easy to exceed linear range of radioactive assay necessitating an expensive and tedious repeat.
  - throughput is modest for radioactive versions tolerable for small numbers of ELISA
  - ELISA assays are VERY tedious to perform without plate washers and dispensers. With them it is very time consuming
  - cost
    - radioactive assays cost >$3.00 each
    - ELISA version ~$2.00/assay (well) or ~$200/96-well plate
    - we do ~10-20 96-well plates/week so this is not reasonable.
Reporter genes 2 - luciferase

- luc gene encodes an enzyme that is responsible for bioluminescence in the firefly. This is one of the few examples of a bioluminescent reaction that only requires enzyme, substrate and ATP.
- Rapid and simple biochemical assay. Read in minutes
- Two phases to the reaction, flash and glow. These can be used to design different types of assays.
  - Addition of substrates and ATP causes a flash of light that decays after a few seconds when [ATP] drops
  - after the flash, a stable, less intense “glow” reaction continues for many hours - AMP is responsible for this

Flash reaction

Glow reaction
Reporter genes 2 - luciferase (contd)

- flash reaction is ~20x more sensitive than glow
  - 5 fg of luciferase or subattomolar levels (10^{-18} \text{ mol})
  - substrate must be injected just before reading (equipment requirement)
  - OR stabilized assay utilized (5’1/2 life) This uses CoA (increased cost)

- glow reaction is more stable
  - allows use of scintillation counter
  - no injection of substrates required
  - potential for simple automation in microplate format
    - add reagents, read at leisure
Reporter genes 2 - luciferase (contd)

• advantages
  – large dynamic range up to 7 decades, depending on instrument and chemistry
  – rapid, suitable for automation
  – instability of luciferase at 37 °C (1/2 life of <1hr) improves dynamic range of transient assays
    • at least one vendor has stabilized luciferase by removing the peroxisome targeting signal - lower dynamic range
  – inexpensive - <$0.40/reaction commercially or <$0.05 homemade
  – this is <$5.00/96-well plate - reasonable
  – widely used

• disadvantage is equipment requirement
  – luminometer (very big differences between models)
    • photon counters - very sensitive, saturate rapidly (~100,000 events/second) 5 decades or so
    • induced current - do not saturate but may not be as sensitive (5 decades)
    • a very few are sensitive and have large linear range (6-7 decades)
  – OR liquid scintillation counter (photon counter)
**Reporter genes 3 - Renilla luciferase**

- isolated from the sea pansy Renilla reniformis (occurs off CA coast)
- different substrate coelenterazine
  - very expensive $80/mg vs $0.99 for luciferin
  - commercial assays - dual luciferase $0.75/reaction
  - ~$75/96-well plates
- requires a luminometer that can do multiple injections
  - or must use glow reactions
- typically used as transfection control, rather than true reporter gene due to expense
- no advantages over standard luciferase as reporter
- slight advantage as transfection control (fast, sensitive)
Reporter genes 4 - β-galactosidase

- very stable enzyme tetramer
- cleaves β-D galactoside linkage
- simple biochemical reaction
  - but must take care to stay in linear range
- detection sensitivity depends on substrate used in enzymatic assay (fast, inexpensive)
  - colorimetric - ONPG, ~500 pg/ml, <$0.001/rxn
  - fluorescent - MUG ~50 pg/ml
  - chemiluminescent ~20 fg/rxn, $0.70/rxn
- OR ELISA substrate used (slow, very expensive) ~50 pg/ml ~$2.00/rxn
  - colorimetric
  - fluorescent
  - chemiluminescent
Reporter genes 4 - β-galactosidase (contd)

- **dynamic range**
  - enzymatic assays are 3 (colorimetric) to 5 (chemiluminescent) decades.
  - ELISA assays - 3 decades

- **advantages**
  - can be very inexpensive
  - can require little equipment (spectrophotometer)
  - stable enzyme at 37ºC - good for embryos

- **disadvantages**
  - sensitive assays are expensive and time consuming (ELISAs) or require considerable equipment
    - luminometer
    - fluorometer
  - stability of the enzyme makes it a poor choice for reporter in transient transfections (high background = low dynamic range)
  - variable background from endogenous β-galactosidases
  - may not function in some cell types (e.g. Xenopus cells)

- **primary applications**
  - frequently used as a transfection control
  - reporter in transgenic animals
  - lineage tracer in microinjected embryos
Reporter genes 5 - β-glucuronidase

- very stable enzyme tetramer similar to β-gal
- cleaves β-D glucuronide linkage
- simple biochemical reaction
  - but must take care to stay in linear range
- detection sensitivity depends on substrate used in enzymatic assay (fast) but similar to β-gal
  - colorimetric and fluorescent substrates available
- dynamic range - 3 decades
- advantages
  - low background
  - can require little equipment (spectrophotometer)
  - stable enzyme at 37°C
- disadvantages
  - sensitive assays require
  - stability of the enzyme makes it a poor choice for reporter in transient transfections (high background = low dynamic range)
- primary applications
  - typically used in transgenic plants with X-gus colorimetric reporter
Reporter genes 6 - β-lactamase

- based on E. coli bla gene - cleaves β-lactam rings in penicillins and cephalosporins
- load up living cells with CCF2/AM reagent and monitor change in fluorescence from 520 nm to 447 nm in a fluorometer
- sensitive detection.
  - Possible to detect activity in single cells with stably transfected reporter cells
  - sensitivity is femtomolar (about 1000x less than luciferase)
- dynamic range is ~ 6 decades
- primary use is for reporter in high throughput assays using living cells
Reporter genes 6 - β-lactamase (contd)

• advantages
  – single cell detection allows FACS sorting of transfected cells
  – can use as an insertional trap in living cells
    • random insertions into genome can be mapped to genes and analyzed
  – sensitive detection

• disadvantages
  – expensive, single source (Aurora Biotech)
  – license limitations (<1000 compounds/year)
  – equipment requirement - fluorometer or fluorescence microscope equipped for FRET analysis
  – not yet widely used
Reporter genes 7 - human growth hormone (hGH)

- a relatively old and not widely used reporter system that employs human growth hormone as a secreted reporter gene
  - kits sold by Nichols Institute - local
- can be used in living cells
- ELISA assay or RIA
- sensitivity
  - ELISA - 5 pg/ml
  - RIA - 100 pg/ml
- linearity is ~2-3 decades
- expensive ~$2/rxn
- advantage
  - measure activity in supernatant
  - kinetics possible
  - sensitive
- disadvantage
  - expensive - unthinkable for large scale transfections (~$200/96-well plate)
  - ELISA assay is time consuming and tedious
  - stability of GH in medium gives the assay only a very modest dynamic range
Reporter genes 8 - secreted alkaline phosphatase (SEAP)

- reporter construct produces human placental alkaline phosphatase that is secreted into the medium
- very simple biochemical assay. Simple addition of substrate to aliquot of culture medium.
- Sensitivity is ~10 fg/assay (attomolar levels)
- linearity is ~5 decades
- moderately expensive ~$0.40/rxn
- advantages
  - cell lysis not required
  - can monitor gene expression over time or in response to changing treatments
  - can monitor kinetics of response
- disadvantages
  - not widely used (reviewers)
  - need to construct new reporters
- applications
  - much better for stable cell lines

**Figure 25. Principle of the SEAP Reporter Gene Assay, chemiluminescent.** Alkaline phosphatase is secreted into cell culture medium (step 1). The secreted alkaline phosphatase (SEAP) dephosphorylates CSPD substrate, emitting light at 477 nm (step 2).
Reporter genes 9 - green fluorescent protein (GFP)

- source is bioluminescent jellyfish Aequora victoria
  - GFP is an intermediate in the bioluminescent reaction
- absorbs UV (~360 nm) and emits visible light.
  - has been engineered to produce many different colors (green, blue, yellow, red)
  - These are useful in fluorescent resonance energy transfer experiments
- simply express in target cells or embryos and detect with fluorometer or fluorescence microscope
- sensitivity is low
  - GFP is non catalytic, 1 µM concentration in cells is required to exceed autofluorescence
- dynamic range is modest ~ 3 decades
- advantages
  - can detect in living cells
    - kinetics possible
    - lineage tracing possible
    - FACS analysis possible
  - inexpensive (no substrate)
- disadvantages
  - low sensitivity and dynamic range
  - equipment requirements
- primary applications
  - lineage tracer and reporter in transgenic embryos
Reporter genes summary - which reporter is best?

- What is the sensitivity required?
  - Luciferase and β-lactamase are most sensitive
- What dynamic range is needed?
  - Luciferase and β-lactamase have 6-7 decades
- What is the cost of the enzymatic substrate vs the sensitivity required?
  - Luciferase is by far the least costly of the sensitive assays
  - Colorimetric β-gal is the cheapest overall
- How much labor is required to perform the assay vs the cost/assay
  - GFP is the easiest - no substrate or reaction
  - Luciferase and β-lactamase are good choices
  - ELISA based assays are expensive and painful
- How many assays are required in what time period?
  - Luciferase and enzymatic β-gal are the fastest
- How convenient is it to do required transfection controls?
  - Luciferase and β-gal is cost effective
  - β-lactamase may be best for stables
Reporter genes summary - which reporter is best? (contd)

- What is the equipment required?
  - β-lactamase requires most costly equipment (tunable fluorometer) (~$40-120K)
  - luciferase requires a good luminometer for high sensitivity (~$20K)
    - scintillation counter is less sensitive and more expensive ($35-$80K)
  - ELISA assays require plate readers (washers and dispensers are necessary for high throughput)

- Is in vivo detection required?
  - β-lactamase is most sensitive
  - GFP is a good choice for lineage tracing

- What equipment and reagents are readily available vs when you need to do the assays?
  - It doesn’t make sense to set up a new method if you need to move quickly and something is already working in the lab
  - Is the equipment available (e.g. luminometer)

- What is already working in the lab vs projected cost, sensitivity and throughput issues?
  - E.g. luciferase is the most cost effective overall but this may not be the case if you already have CAT reporters and don’t need to do many assays