Technical tips
Session 4

Biotinylation assay: Streptavidin is a small bacterial protein that binds with high affinity to the vitamin biotin. This streptavidin-biotin combination can be used to link molecules such as proteins that normally do not interact. Proteins can be labeled with biotin through the formation of an amide bond between the biotin and free amino groups present in lysines.

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There are many different uses for this kind of assay: for example, the streptavidin can be conjugated to an enzyme such as HRP (horseradish peroxidase), which catalyzes a luminescent reaction in the presence of the substrate luminol (see technical tips for session 3). You can then visualize a signal in a Western blot by biotinylation of the primary antibody and further use of streptavidin-HRP instead of a secondary antibody. Alternatively, you can biotinylate a protein and recognize streptavidin-HRP bound to it with an antibody that recognizes the HRP.

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(In Mosesson *et al.*, 2003, they use the biotinylation assay to check whether the protein EGFR is at the cell surface (they do the assay with whole cells, not lysates or cell extracts). Biotin won't have access to protein inside the cells so only external EGFR will be biotinylated. Then, EGFR is immunoprecipitated with specific antibodies. To detect whether EGFR has been biotinylated they run the immunoprecipitates in SDS-PAGE and do Western using a primary antibody that specifically recognizes streptavidin-HRP).

Transient transfection: The introduction of genetic material (usually DNA) in microorganisms such as bacteria and yeast is called *'transformation'*. In these organisms naked exogenous DNA can be introduced in the cells by treatments that make holes into the cells membranes without killing them. If the DNA has their own replication sequences and some gene ('marker') that allows the cells to survive under certain conditions, it can be maintained in the cells for many generations (as long as in the presence of such selective conditions). Moreover, DNA sequences can also be integrated into the genome by using homologous recombination. In higher eukaryotic cells (such as ours) foreign DNA cannot be as easily introduced and different systems have been ideated to allow the expression of foreign genes on them. These systems often make use of chemically or physically inactivated viruses (in which their pathogenic properties have been eliminated) as vectors for introduction of exogenous DNA. This method is called *'transfection'*. In a *'transient transfection'* the DNA, once inside the cell, stays in the cytosol as an autonomous entity (like a *plasmid*) where it express itself for a short period of time (usually about 48 hours) but is eventually lost because of its failure to be inherited by daughter cells. In a *'permanent transfection'* the DNA would integrate inside the cell's genome so it would be perpetually inherited by the next generations. In the first case the viral system is only used for the entrance of the genetic material inside the cells but in the second case viral sequences (such as that of retroviruses) capable of genomic integration are also used to insert the exogenous DNA into the cell's genome.

BIAcore biosensor: Optical biosensor, instrument that can define the assembly state, thermodynamics and kinetics of an interaction. There are three main components to all affinity-based biosensors. First, it has a

detector that uses *SPR* (*surface plasmon resonance*), electron waves created at metal surfaces when biomolecules are attached to the surface. These waves can be used to monitor binding reactions because of changes in refractive index of the solvent near the surface. Second, there is a *sample delivery system*, which rapidly introduces and washes away analyte during a binding experiment. Third, it has a *sensor surface*, where the ligand may be immobilized using a variety of coupling methods.

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Sucrose cushion pelleting: Method to separate particles from cell extracts such as cell organelles (membranous compartments, vesicles, etc) on the basis of their buoyant (isopycnic) density or their rate or velocity of sedimentation. A gradient of density can be created in a centrifuge tube by using different concentrations of the polysaccharide sucrose. When you add your sample to it and centrifuge at high-speed, molecules/organelles will be trapped in different fractions in which their density is equal.
(In Pornillos *et al.* 2003, they previously separate the cell's fraction by centrifuging at low speed and then they obtain the viral particles released to the medium by pelleting them (from the supernatant of the first centrifugation) by centrifugation at high speed in a sucrose gradient).

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siRNA and the Mechanism of RNA Interference (RNAi): Long double-stranded RNAs (dsRNAs; typically >200 nt) can be used to silence the expression of target genes in a variety of organisms and cell types (e.g., worms, fruit flies, and plants). Upon introduction, the long dsRNAs enter a cellular pathway that is commonly referred to as the RNA interference (RNAi) pathway. First, the dsRNAs get processed into 20-25 nucleotide (nt) small interfering RNAs (siRNAs) by an RNase III-like enzyme called Dicer (initiation step). Then, the siRNAs assemble into endoribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs), unwinding in the process. The siRNA strands subsequently guide the RISCs to complementary RNA molecules, where they cleave and destroy the cognate RNA (effecter step). Cleavage of cognate RNA takes place near the middle of the region bound by the siRNA strand.

In mammalian cells, introduction of long dsRNA (>30 nt) initiates a potent antiviral response, exemplified by nonspecific inhibition of protein synthesis and RNA degradation. The mammalian antiviral response can be bypassed, however, by the introduction or expression of siRNAs.

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Gene Specific Silencing by RNAi: RNA interference (RNAi) is a phenomenon in which the introduction of double-stranded RNA (dsRNA) into a diverse range of organisms and cell types causes degradation of the complementary mRNA (Figure 1; step 1). In the cell, long dsRNAs are cleaved into short 21-25 nucleotide small interfering RNAs, or siRNAs, by a ribonuclease known as Dicer (step 2). The siRNAs subsequently assemble with protein components into an RNA-induced silencing complex (RISC), unwinding in the process (step 3). Activated RISC then binds to complementary transcript by base pairing interactions between the siRNA antisense strand and the mRNA. The bound

mRNA is cleaved (step 4) and sequence specific degradation of mRNA (step 5) results in gene silencing.

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Site-directed mutagenesis: Mutagenesis where the mutation is caused by in vitro induction directed at a specific site in a DNA molecule. The earliest methods involve the use of a chemically synthesized oligonucleotide mutant sequence that can hybridize with the DNA target molecule. The resulting mismatch-carrying DNA duplex is then introduced into a bacterial cell line and bacterial polymerases use this DNA duplex and the native strand to regenerate the missing strand. The newly-synthesized strand will contain the mutation on it.

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But the most common method used today utilizes PCR. The basic procedure also utilizes a supercoiled double-stranded DNA (dsDNA) vector with an insert of interest (sequence you want to mutate, such as a gene) and two synthetic oligonucleotide primers containing the desired mutation. The oligonucleotide primers, each complementary to opposite strands of the vector, are extended during temperature cycling by the DNA polymerase. Incorporation of the oligonucleotide primers generates a mutated plasmid containing staggered nicks. Following temperature cycling, the product is treated with *Dpn* I. The *Dpn* I endonuclease is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesized DNA (DNA isolated from almost all *E. coli* strains is dam methylated and therefore susceptible to *Dpn* I digestion). The nicked vector DNA containing the desired mutations is then transformed back into the bacteria where enzymes repair the nick.

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