5/19/10  Direct Detection of Genotype

about gene testing:
http://www.ornl.gov/sci/techresources/Human_Genome/medicine/genetest.shtml

about sickle cell:
http://ghr.nlm.nih.gov/condition=sicklecellanemia
http://www.ygyh.org/
A glu → val missense mutation in the β hemoglobin gene causes sickle cell anemia

Loss-of-function mutations cause β thalassemias (form of anemia)
You are a clinical geneticist and you want to
• determine the genotype of an individual at a particular locus -- *such as the β globin gene*
• or study a *the β globin gene* in a group of individuals

You have a tube of genomic DNA prepared from spit or blood or whatever.

It is a complex heterogeneous mixture of sequences

How are you going to examine your gene of interest?
**PROBLEM:**
- β hemoglobin gene 3 kb/3 million kb: *1 part in 1 million of the genome*
- Small quantity of your gene and it’s contaminated with all of these other sequences

**WANT A TUBE LABELED:**

*PURE β hemoglobin sequences*
How to purify the gene sequences that you want to study?

If you were trying to purify a protein what would you do?
Can take advantage of natural systems to amplify specific DNA sequences in vivo (see pg22 of this lecture)

OR

Amplify the gene or sequence using PCR -- an in vitro process

PCR = polymerase chain reaction
• Very sophisticated molecular technologies have developed based on our understanding of the enzymology of DNA replication, transcription and translation
• PCR is an in vitro DNA replication technology that has revolutionized basic research in molecular biology and genetics
• PCR involves exponential amplification of a specific gene or region of DNA from a complex mixture of DNA
How do we target amplification to our specific sequences of interest?

How come only the red sequence is amplified from the starting template:
Specificity of amplification is controlled by the primers added to the reaction—WHY?

If this segment is to be copied...

...the primers have to be chosen from this sequence...

5' - C'TAGAATATGAAACCTATAGGTA CGGGCCATTTCTATGTCTGATCCGGTACTACCTACAGAA-3'

3' - GATCTTTATACCTTGATATCCATGC ACCCGGTAGATACAGACTAGGGCCATGATGGATGTCTT-5'

...and this sequence
What are the components of a PCR Reaction?

- Template DNA
- Gene or sequences to be amplified
- Primers
- PCR product

20 or so cycles
PCR animations
http://www.dnalc.org/ddnalc/resources.animations.html

See pg 23 for more detailed schematic

What is temperature scale in °C?

Melt = denature DNA with heat
Anneal = allow primer to hydrogen bond with complementary sequences on the template DNA
Replicate = allow DNA polymerase to extend primer and synthesize complementary copy of template
The complete sequence of human beta globin gene is shown below

Conventions for displaying gene sequences:
- Sequence reads 5’ to 3’
- Only the mRNA like strand is displayed (complementary strand not shown)

>ref|NG_000007.3|:70000-73000 Homo sapiens beta globin (HBB) on chromosome 11

CACACATATATATATATATTTTTTCTTTTCTTTTCCACAGAGGTGTTTTTAAATCCAATATAGGAGAGATATGCTT
TAGACCGAGGTAGAGTTTCTACATCTTGCCCTGCTGCTAAGTATTTGGAGACGAGAAAGAAGATCCATCTATATCC
GATCCATCTCATATCCCAAAAGTCGAAATTATGGAAGACAAACTCTTCACCTTTAGTGATCAACTCTCT
TATTGTGTAATAAGAAAATTTGGGAAAACGATCTTCAATATGCTTACCAAGCTGTGATTCCAAATATTAG
GCTTGTAATCATGCAAGGAGAGTTTCTTTGTAAGTTAGCTAGTATGATGATAAGGCGCAAGAGATATA
TCTTAGAGGGAGGGCTGAGGGTTTGAAGTCCAACTCCTAGCCAGTGCCAGAAGAGCCAAGGACAGGTAC
GGCTGTCATCACTTAGACCTACCTCCCTGTCGACCGCAACCTAGCTGGCCTTAACTCAAGGAGCAG
GAGGGCAGGCAGCCAGGCTGGGC

In blue: transcription start site
Highlighted ATG: start of translation

TATA box – part of the promoter: mutations here cause loss-of-function phenotype (thalassemia)

Sickle cell mutation
CCT-GAG-GAG  CCT-GTG-GAG.
Design primers to amplify a region spanning the sickle cell mutation --
between the ///’s but not extending beyond the symbols in either direction:
• Both primer sequences must read in the 5’ to 3’ direction.
• Primers are typically 20-30 bases long

>ref|NG_000007.3|:70000-73000 Homo sapiens beta globin (HBB); and hemoglobin on
chromosome 11
CACACATATATATATATATATTTTTTCTTTTCTTACCAGAAGGTTTTTAATCCAAATAAGGAGAAGATAT
GCTTAGAACCGAGGTAGAGTTTTTCATCCATTCTGCTCTGTAAGTATTTTTGATATTTCTGGAGACGCA
GGAAGAGATCCATCTACATATCCAAAAGCTGAATTATGCTAGACAAACTCTTCCACTTTTAGTGCAC
TCAACTTTCTTTTATTTGTGAATAAGAAATTGGAACACCAGATCTTTCAATATGCTTACCAAGCTGTGAT
TCCAAATATTACGTAAATACACTTGCAAGAGAGGATGTTTTTTAGTAGCAATTGATACTGTAGGTATG
GGGCCAAGAGATATATCTTTAGAGGGAGGCTAGGTTTTGAACACTTCAAGGCAGCTGCGAG
AGAGCCAAGGACAGGTACGGCTGTACATCAGTTAGAACACTCTACCTCAGCTTGAG
GGCCAATCTACTCCAGAGCAGGGGAGGCAAGGACAGCCATTGGGCATAAAAGTCAGGGGAGACCA
ATCTATTGCTTTAGATTTTGCTTCTGACACAAACTGTGTTTCACTAGCAACCTCAAGACAGACCAGGTGTG
CATCTGACTCATTGAGGAGTCTGCGTTACTGCGCTTGTGGGCAGGTGAACGTGATGAGTGGT
GTGTGAGGCCCTTGGCAGGTTGATTATCAAGGACACAGGTTATAGGAGAGACAGCAGAGGGCTG
GGCATGTTGGAAGACAGAAGACTCTTTTCTGGTATAGGCACTGACTCTCTCCTGGCTTATTGGTCTTA
TTTTTCCCACCCCTTACCGCCTGCTGCTGGTGTTCTACCTTGGGAGCCAGGAGTTCCTTGGT
ATCTGTCCACTCCTGATGCTGTGTATGGAAGCCTAGGCAAAGCTGCTGGCTGGGCAGCAGAGGCTG
TGCCCTTTAGTGATGGCCTTGGCCTACCTGGAAGACACCTCAAGGGGACCCCTTGGCACAAGTGAGTGA
CAGTGTG
Left primer: 5’ GAGCCACACCCCTAGGGTT etc.
Right primer: 5’ CAAAGAACCTCTCTGGGT etc.
What do you do with your PCR product once you make it?

How can you determine genotype using your PCR product?

How do we know if the globin sequence is

CCT-GAG-GAG VS CCT-GTG-GAG
**Restriction enzyme** = *restriction endonuclease*

- *nuclease* = enzyme that hydrolyzes phosphodiester bond
- *endo* = cuts internally in the DNA polymer (vs. exo that cleaves from the end
- *restriction* = nuclease activity restricted to a site with a specific DNA sequence
The A → T transversion mutation in the sickle-cell allele removes an Mst II restriction site.

Mst II recognizes CCTNAGG where N = A, C, G or T.

Note that at this level of examination the two alleles are codominant.
The **SCN9a** gene codes for a subunit of a neuronal sodium ion channel involved in the transmission of pain signals

**On left:** dideoxy sequence display of PCR Product*

**On right:** restriction digest of product of PCR targeting the SCN9A gene

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**Figure 3 Mutations in SCN9A in patients with primary erythermalgia.**

(A) Affected members in the family are heterozygous for a T2573A mutation.

(B) Restriction endonuclease Hph I digestion for the identification of the T2573A mutation in the family. Mutation T2573A abolishes a recognition site for restriction endonuclease Hph I. Three fragments of 470 bp, 303 bp, and 167 bp are found in patients, and two fragments of 303 bp and 167 bp fragments are found in unaffected members.

* Read about Sanger dideoxy sequencing in your textbook
R-spondin1 is essential in sex determination, skin differentiation and malignancy
Figure 2 Mutation analysis and transcript analysis. (a) Mutation analysis in family F. The guanine insertion at nucleotide 896 of the NM_001036033 sequence is shown by an arrow. ‘+/−’ refers to an individual homozygous for the insertion, ‘WT/−’ refers to a heterozygote and ‘WT’ refers to an individual lacking the insertion. (b) Mutation analysis in individual AN. The locations of primers for five separate PCRs are indicated by arrows (3, 4a, 4b, 5 and del). The 4a and 4b amplifications are included in the AN deletion. The primers for the ‘del’ reaction could not amplify the genomic region in control DNA, as the expected size is too large for the applied PCR conditions. Exon (‘Ex’) and intron sizes, as well as primer localization, are not to scale. (c) Genomic structure and alternative splicing in RSP01. The guanine insertion in family F is shown by F*. The deletion in individual AN of family A is indicated by ‘AN del’. 3P, signal peptide; FU, furin-like repeat; TSP, thrombospondin domain; NLS, nuclear localization signal. (d) RT-PCR analysis of the splicing variants of RSP01 found in human adult ovary. The cDNA was amplified with primers RT2F and RT5R (see c). (e) Presence of stable RSP01 transcript in fibroblasts of individual AN. Amplification was performed using primers RT2F and RT5R. Presence of a stable transcript was confirmed with other primers (data not shown).
a. Mutation analysis in family F.

The guanine insertion at nucleotide 896 of exon 5 is shown by an arrow.

‘WT’ refers to an individual lacking the insertion.

‘WT/–’ refers to a heterozygote.

‘–/–’ refers to an individual homozygous for the insertion.
(b) Mutation analysis in individual AN. The locations of primers for five separate PCRs are indicated by arrows (3, 4a, 4b, 5 and del). The 4a and 4b amplifications are included in the AN deletion. The primers for the ‘del’ reaction could not amplify the genomic region in control DNA, as the expected size is too large for the applied PCR conditions. Exon (‘Ex’) and intron sizes, as well as primer localization, are not to scale.
Taking advantage of natural systems to amplify specific DNA sequences

Use recombinant DNA techniques to generate a molecular clone of the DNA: use a cell such as *E. coli* to make lots of copies of your gene -- put it in a DNA molecule that is easy to recover in a pure form from the cell.
(A) Target DNA to be amplified

1. Denature by heating

2. Allow added oligonucleotide primers to anneal

3. DNA synthesis

No. of DNA strands

- 1st cycle
- 2nd cycle
- 25th cycle

5' 3'