BIO-RAD

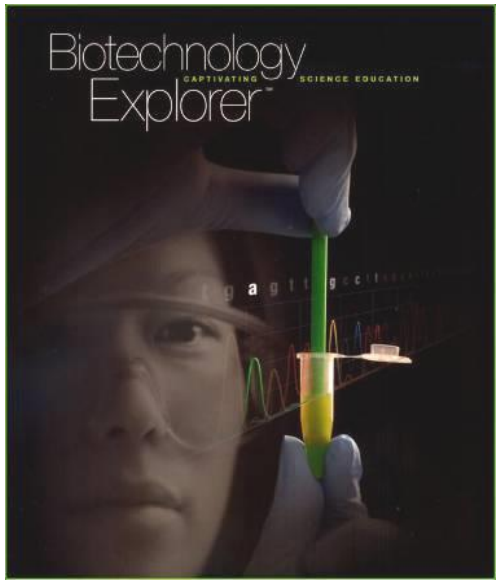
Professional Development

Biotechnology Explorer™ CAPTIVATING SCIENCE EDUCATION

BIO-RAD

Novel Research Starts with… *GAPDH*

Instructors



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Why Teach

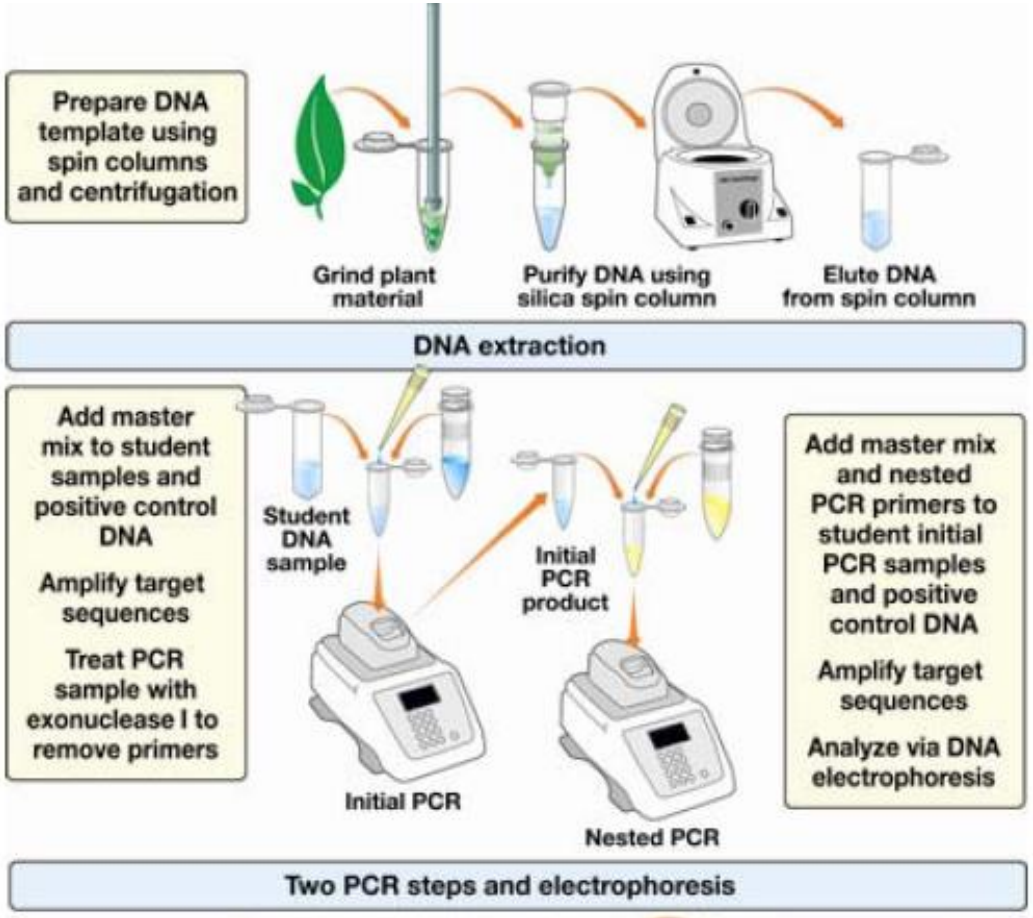
Novel Research Using *GAPDH* PCR?

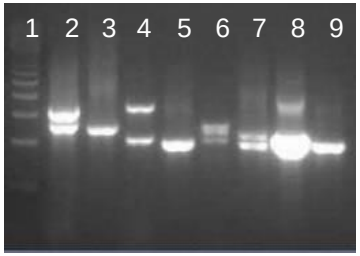
- Students conduct inquiry-based experiments
- Students understand research is a process rather than a single experiment
- Students learn laboratory skills and techniques commonly used in research
- Students guide the research process and make decisions about their next steps
- Students formulate scientific explanations using data, logic, and evidence
- Students learn from failures and unexpected results

Workshop Timeline

- Introduction
- Preparation of Initial PCR Reactions
- Exonuclease Treatment
- Preparation of Nested PCR Reactions
- Analyze PCR results

Laboratory Overview

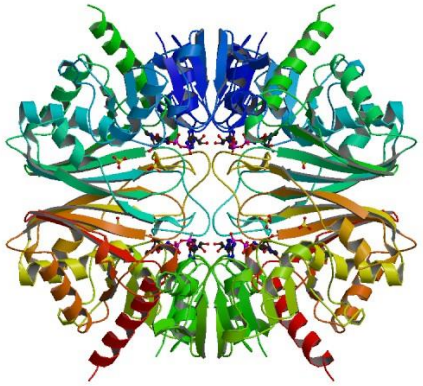




Benefits of using plants

- Large number of species
- Lots of diversity
- Phylogenetic approaches
- Avoid ethical concerns associated with animals
- No pre-approval

What is a Housekeeping Gene?



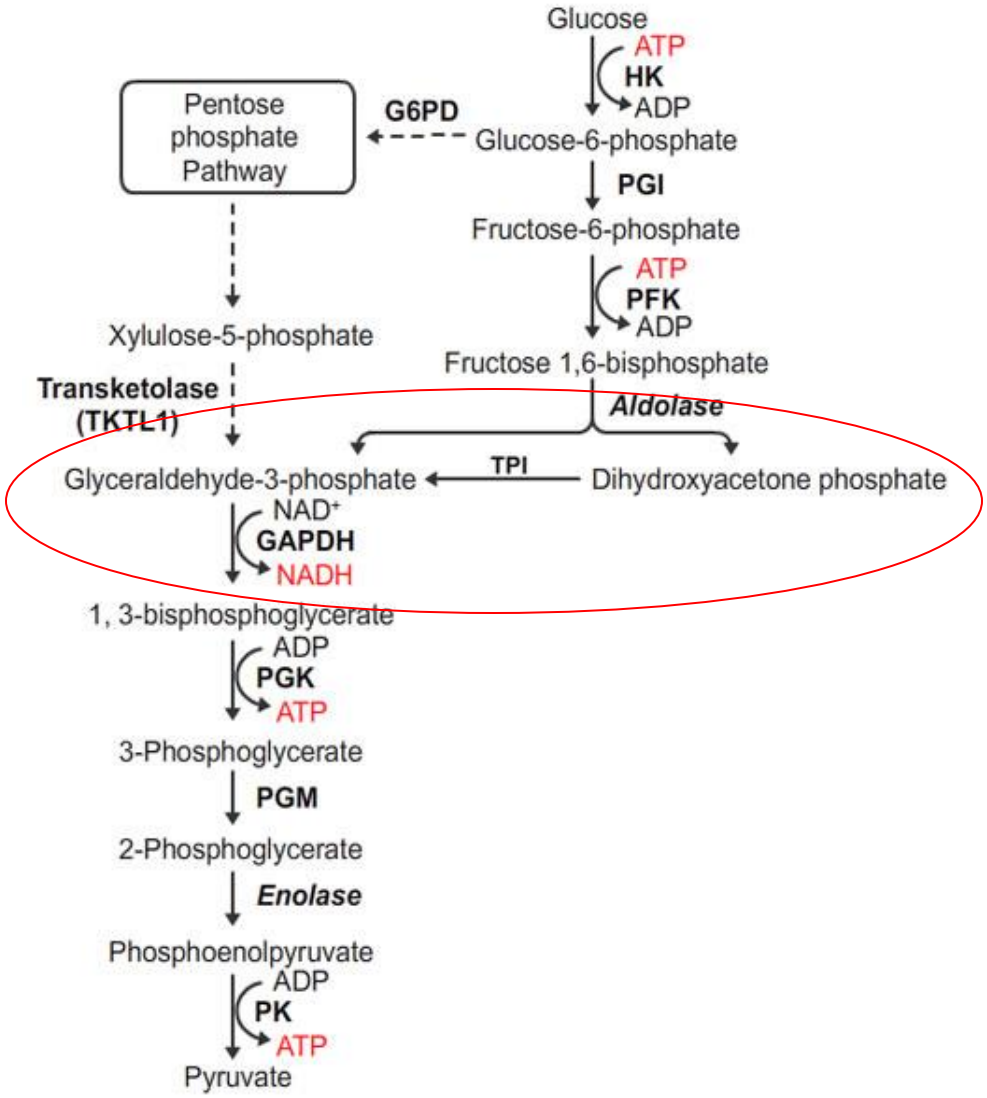
Highly conserved genes that must be continually expressed in all tissues of organisms to maintain essential cellular functions.

Examples:

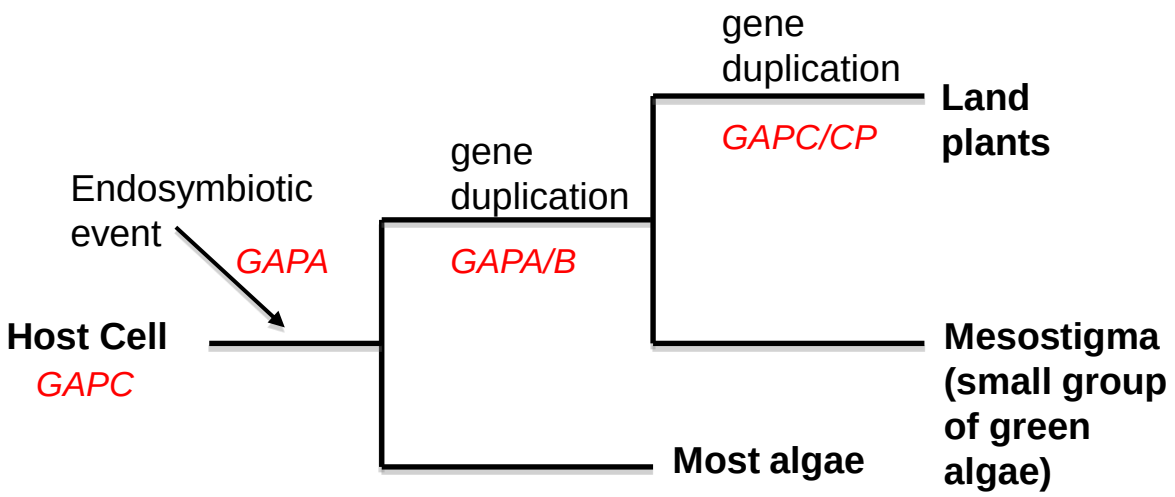
- ***GAPDH***
- ***Cytochrome C***
- ***ATPase***
- ***ß-actin***

Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH)

- **Enzyme of glycolysis**
- **Structure and reaction mechanism well-studied**
- **Multitude of sequences**
- **Highly conserved**



Gene Families

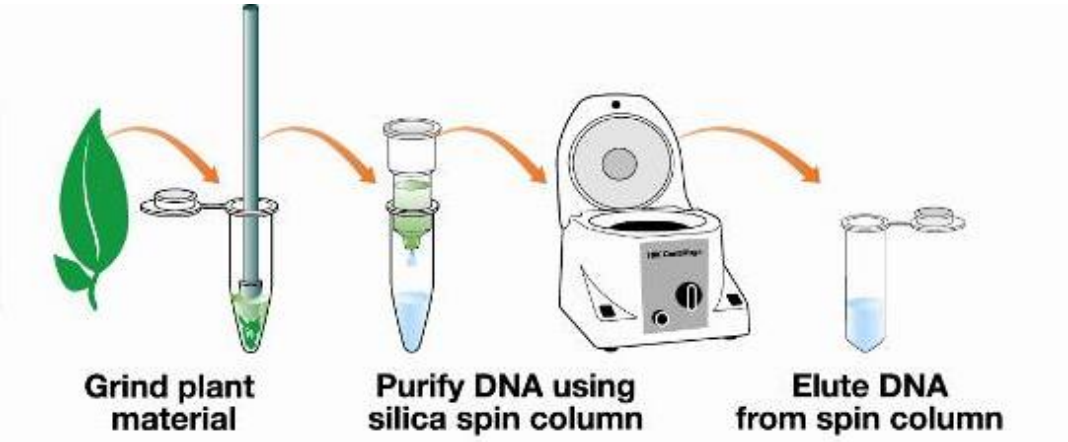


Enzyme Function	GAPDH Protein Subunit	EC Designation*	Arabidopsis Gene
NAD^+-dependent GAPDH in cytosol	GAPC	EC 1.1.1.12	GAPC
	GAPC-2		GAPC-2
NAD^+-dependent GAPDH in plastids	GAPCP	EC 1.1.1.12	GAPCP
	GAPCP-2		GAPCP-2

Extracting DNA

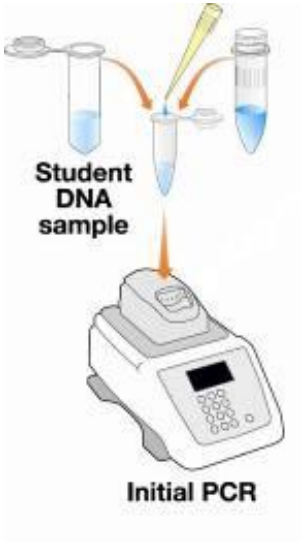
How do we isolate and Identify the *GAPDH* gene?

- **Use young, fresh plant-tissue**
- **DNA extraction at room temperature**
- **Time requirement ~30 minutes**
- **Does not require DNA quantification**

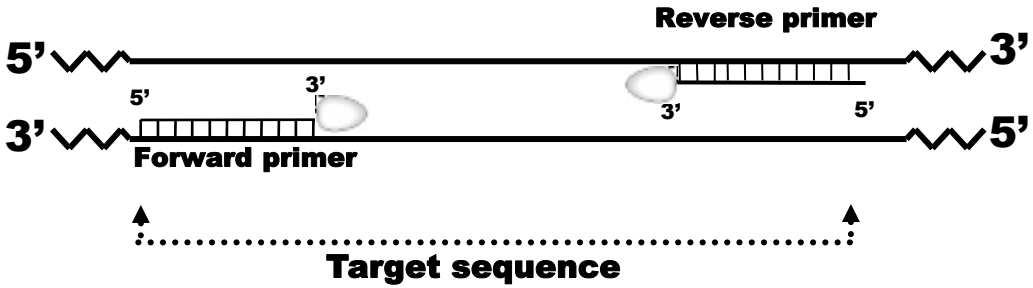


- **DNA can be extracted from the fresh plant tissue and multiple copies of the *GAPDH* gene can be amplified using PCR**

What do we need for PCR?

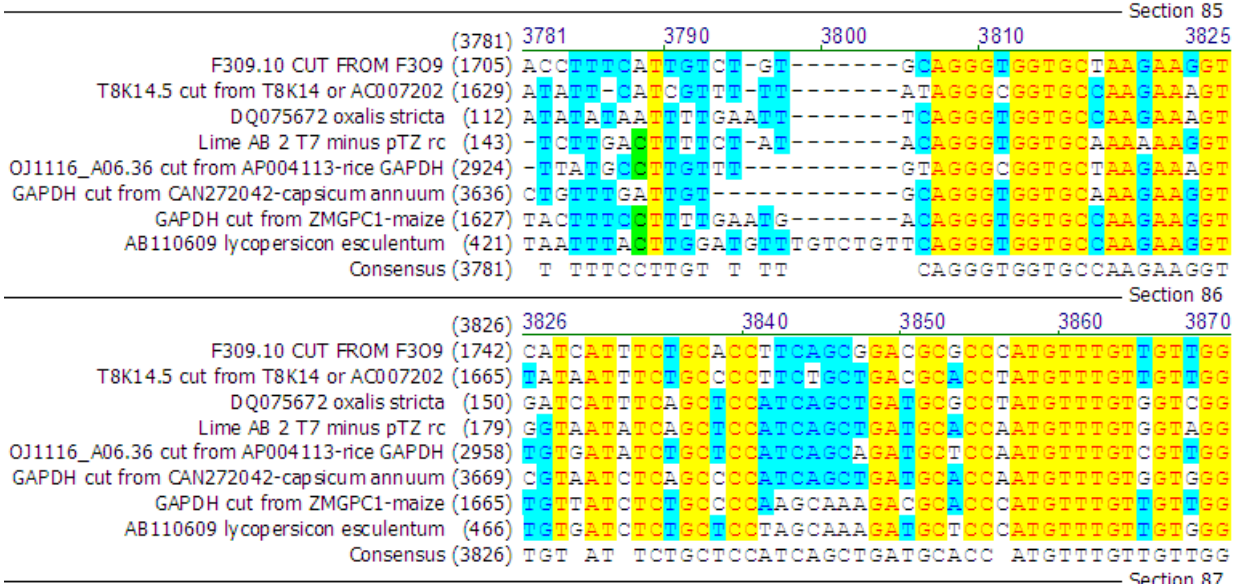


- Template DNA (extracted from plants)
- Nucleotides (dATP, dCTP, dGTP, dTTP)
- Taq DNA polymerase
- Magnesium chloride (enzyme cofactor)
- Buffer, containing salt
- Sequence-specific primers flanking the target sequence

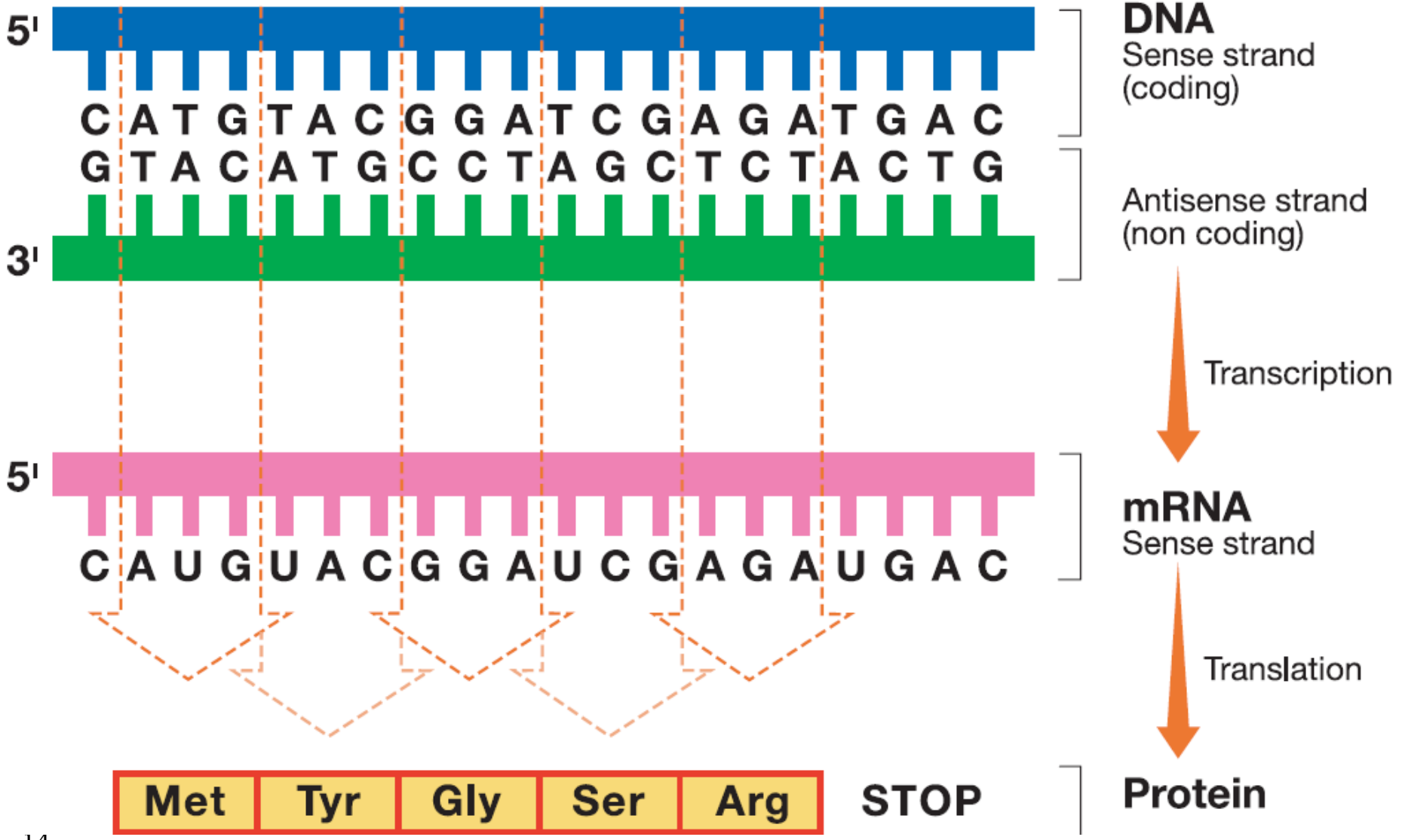


DNA sequence varies between species

Primers need to be designed to account for species variation



- **What if you don't know the exact DNA target sequence?**
- **How do you design primers?**


5'
C A T G T A C G G A T C G A G A T G A C
G T A C A T G C C T A G C T C T A C T G
3'
DNA
Sense strand
(coding)
Antisense strand
(non coding)
Transcription
5'
C A U G U A C G G A U C G A G A U G A C
mRNA
Sense strand
Translation
Met
Tyr
Gly
Ser
Arg
STOP
Protein

Genetic code Degeneracy

- **Multiple codons code for one amino acid**
- **Variations at the DNA level may not be reflected at the protein level**

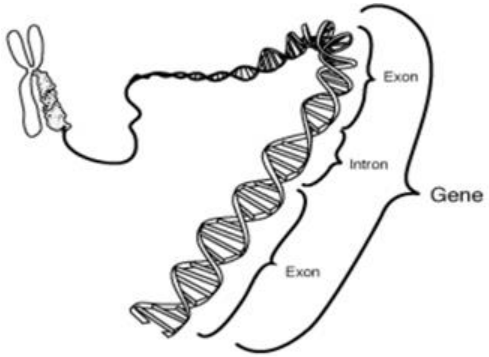
Amino acids and the DNA codons for each.

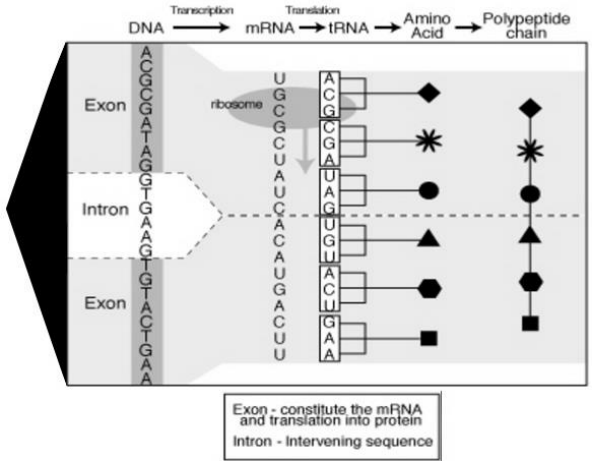
Ala (A)	Arg (R)	Asp (D)	Asn (N)	Cys (C)	Gln (E)	Glu (Q)	Gly (G)	His (H)	Ile (I)
GCA	CGA	GAC	AAC	TGC	CAA	GAA	GGA	CAC	ATA
GCC	CGC	GAT	AAT	TGT	CAG	GAG	GGC	CAG	ATC
GCG	CGG						GGG		ATT
GCT	CGT						GGT		
	AGA								
	AGG								

Leu (L)	Lys (K)	Met (M)	Phe (F)	Pro (P)	Ser (S)	Thr (T)	Trp (W)	Tyr (Y)	Val (V)
CTA	AAA	ATG	TTC	CCA	TCA	ACA	TGG	TAC	GTA
CTC	AAG		TTT	CCC	TCC	ACC		TAT	GTC
CTG				CCG	TCG	ACG			GTG
CTT				CCT	TCT	ACT			GTT
TTA					AGC				
TTG					AGT				

Note: When there are multiple codons for an amino acid, the codons are very similar. For all amino acids with up to four codons, only the third base differs between codons (for example, the four codons for valine, which all begin with GT). Also three codons code for a stop signal, these are TAG, TGA, TAA.

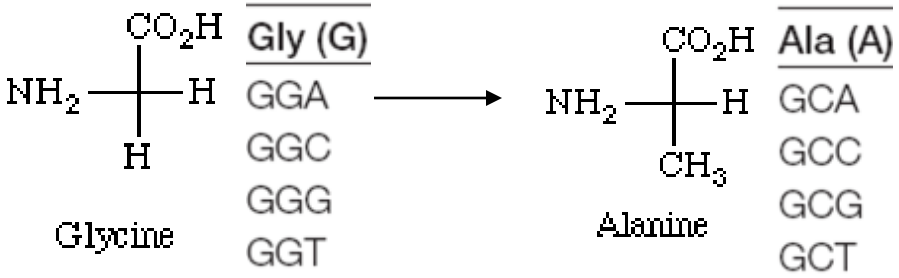
Variation in the Genetic Code





Introns are less conserved since they do not code for protein

The GAPDH enzyme (protein) is highly conserved but there are variations at the DNA level



Conservative substitution-doesn't change protein properties

Degenerate primers are used to account for sequence variation

Plant	*Gene*	Accession number	Sequence
Arabidopsis	*GAPC1*	AT3G04120	GACTACGTTGTTGAGTCTACTGG
Arabidopsis	*GAPC2*	AT1G13440	GACTTTGTTGTTGAGTCTACTGG
Arabidopsis	*GAPCP1*	AT1G79530	GATTATGTTGTTGAGTCTTCCGG
Arabidopsis	*GAPCP2*	AT1G16300	GAGTATGTTGTTGAGTCTTCAGG
Pepper	*GAPCP*	CAN272042	GATTATGTTGTTGAATCTTCTGG
Liverwort	*GAPC*	AJ246023	GAGTACGTCGTCGAGTCTACCGG
Corn	*GAPC1*	ZMGPC1	GAGTACGTCGTGGAGTCCACCGG
Corn	*GAPC2*	U45855	GAGTATGTCGTGGAGTCCACCGG
Corn	*GAPC3*	U45856	GAATATGTTGTTGAGTCTACTGG
Corn	*GAPC4*	X73152	GAATATGTTGTTGAGTCTACTGG
Pea	*GAPC1*	L07500	GATATCATTGTTGAGTCTACTGG
Wheat	*GAPC*	EF592180	GAGTACGTTGTTGAGTCCACCGG
Rye Grass	*GAPC3*	EF463063	GACTACGTTGTTGAGTCCACTGG
Tobacco	*GAPC*	AJ133422	GATTACATTGTGGAGTCGACTGG
Tobacco	*GAPDH**	DQ682459	GATTTCGTTGTGGAATCCACTGG
Carrot	*GAPDH**	AY491512	GAGTACATTGTGGAGTCCACTGG
Blue Gem	*GAPDH**	X78307	GAGTACGTCGTTGAGTCGACTGG
Tomato	*GAPDH**	AB110609	GACTTCGTTGTTGAATCAACCGG
Snapdragon	*GAPDH**	X59517	GAGTATATTGTGGAGTCCACTGG
INITIAL FORWARD PRIMER			GABTATGTTGTTGARTCTTCWGG
Position of base			123456789

Primers are designed using the consensus sequence

Plant *GAPDH*	Accession Number	Sequence
Tobacco	DQ682459	GATTTCGTTGTGGAATCCACTGG
Carrot	AY491512	GAGTACATTGTGGAGTCCACTGG
Blue gem	X78307	GAGTACGTCGTTGAGTCGACTGG
Tomato	AB110609	GACTTCGTTGTTGAATCAACCGG
Snapdragon	X59517	GAGTATATTGTGGAGTCCACTGG
Consensus sequence		GABTATGTTGTTGARTCTTCWGG

Primer set:
GA(GTC)TATGTTGTTGA(GA)TCTTC(AT)GG
Yield: 12 primers

Reverse primers are designed in the same fashion

Degenerate primers have optional bases in specified positions

IUB Code	Bases	Derivation of IUB Code
N	A/G/C/T	any
K	G/T	keto
S	G/C	strong
Y	T/C	pyrimidine
M	A/C	amino
W	A/T	weak
R	G/A	purine
B	G/T/C	not A
D	G/A/T	not C
H	A/C/T	not G
V	G/C/A	not T

To increase the probability that the primer will anneal to the target DNA, variable bases are designed into the primer.

Multiple oligos comprise the forward primer

GAGTATGTTGTTGA(GA)TCTTC(AT)GG
GATTATGTTGTTGA(GA)TCTTC(AT)GG
GACTATGTTGTTGA(GA)TCTTC(AT)GG

Position 3 has 3 bases

GA(GTC)TATGTTGTTGAGTCTTC(AT)GG
GA(GTC)TATGTTGTTGAATCTTC(AT)GG

Position 15 has 2 bases

GA(GTC)TATGTTGTTGA(GA)TCTTCAGG
GA(GTC)TATGTTGTTGA(GA)TCTTCTGG

Position 21 has 2 bases

3 x 2 x 2 = 12

different oligonucleotides comprising the forward primer

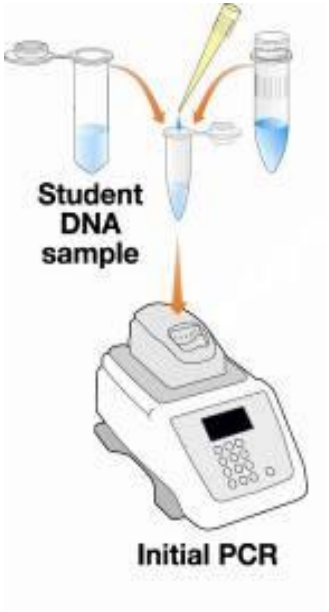
DNA Isolation and Amplification

To identify differences in *GAPDH* code we must isolate plant DNA and amplify the gene of interest using PCR first with degenerate primers (primers that account for variation in the DNA code)

A second PCR reaction (Nested PCR) is necessary to amplify the region which contains one of the *GAPDH* gene sequences (second set of primers are nested inside the initial PCR product sequence)

Biotechnology Explorer PCR primers are color-coded

Setting up initial PCR Reactions



GAPDH PCR – Quick Guide

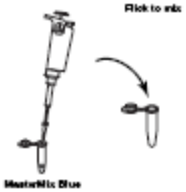
Stage 2A

1. Label five PCR tubes with your initials and the following labels:
   - 1 – Negative control (sterile water)
   - 2 – *Arabidopsis* gDNA
   - 3 – Positive control pGAP plasmid
   - 4 – Plant 1 gDNA (optional)
   - 5 – Plant 2 gDNA (optional)

2. Place each PCR tube into a tube adaptor, cap each tube. Place the adaptor tube with PCR tube on ice.

3. Flick to thoroughly mix reagent tubes and centrifuge 10 sec to force contents to bottom of tubes.

4. Pipet 20 µl of 2x blue mastermix with initial primers (2x MMP) into each PCR tube.



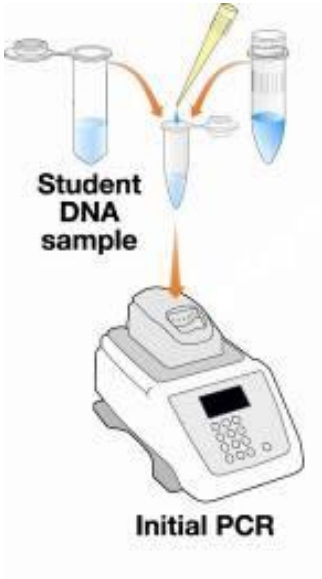
5. Using a fresh tip, pipet 15 µl of sterile water to each tube.

6. Using a fresh tip every time, add 5 µl of the appropriate DNA template to each tube. Gently pipet up and down to mix reagents. Recap tubes.

7. When instructed, place PCR tubes in thermal cycler.

28

Setting up Initial PCR Reactions

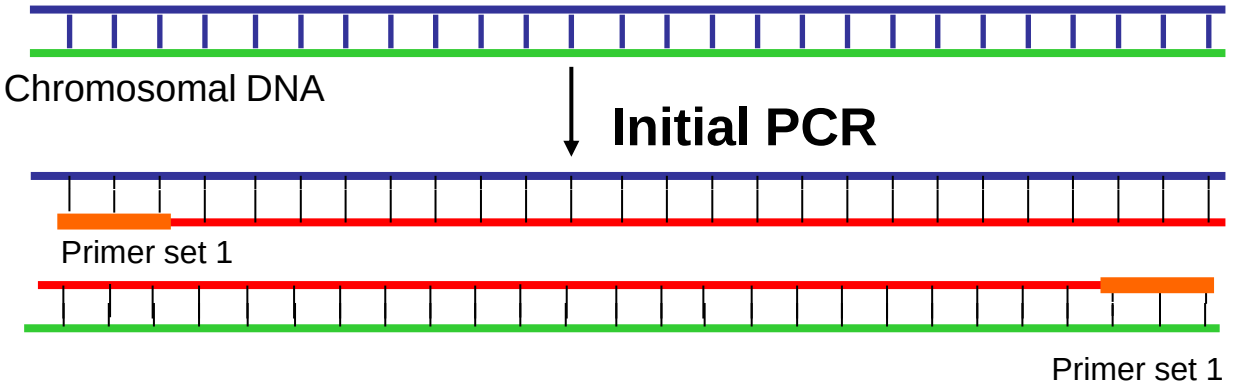


Protocol

1. Add 20 µl of blue mastermix with initial primers to each PCR tube
2. Add 15 µl of sterile water to each tube
3. Add 5 µl of DNA template to the appropriate tube
   - Control Arabidopsis gDNA
   - Control plasmid DNA
   - Test gDNAs
   - Negative control
4. Amplify in thermal cycler (Annealing temp 52°C)

Initial PCR Reaction

(with degenerate primers)



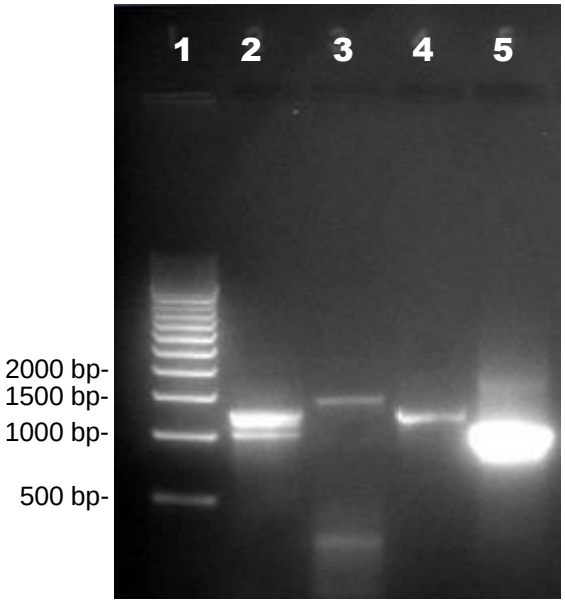
PCR Animation

http://www.bio-rad.com/flash/07-0335/07-0335_PCR.html

Results of Initial PCR Reactions

1% agarose gel loaded with (20 µl) initial PCR samples.

Green bean and Lamb's ear gDNA samples generated using Nucleic Acid Extraction module.



Lane 1- 500 bp molecular weight ruler (10 µl),

Lane 2- PCR of **control *Arabidopsis* gDNA** with initial primers (20 µl)

Lane 3- PCR of **green bean gDNA** with initial primers (20 µl)

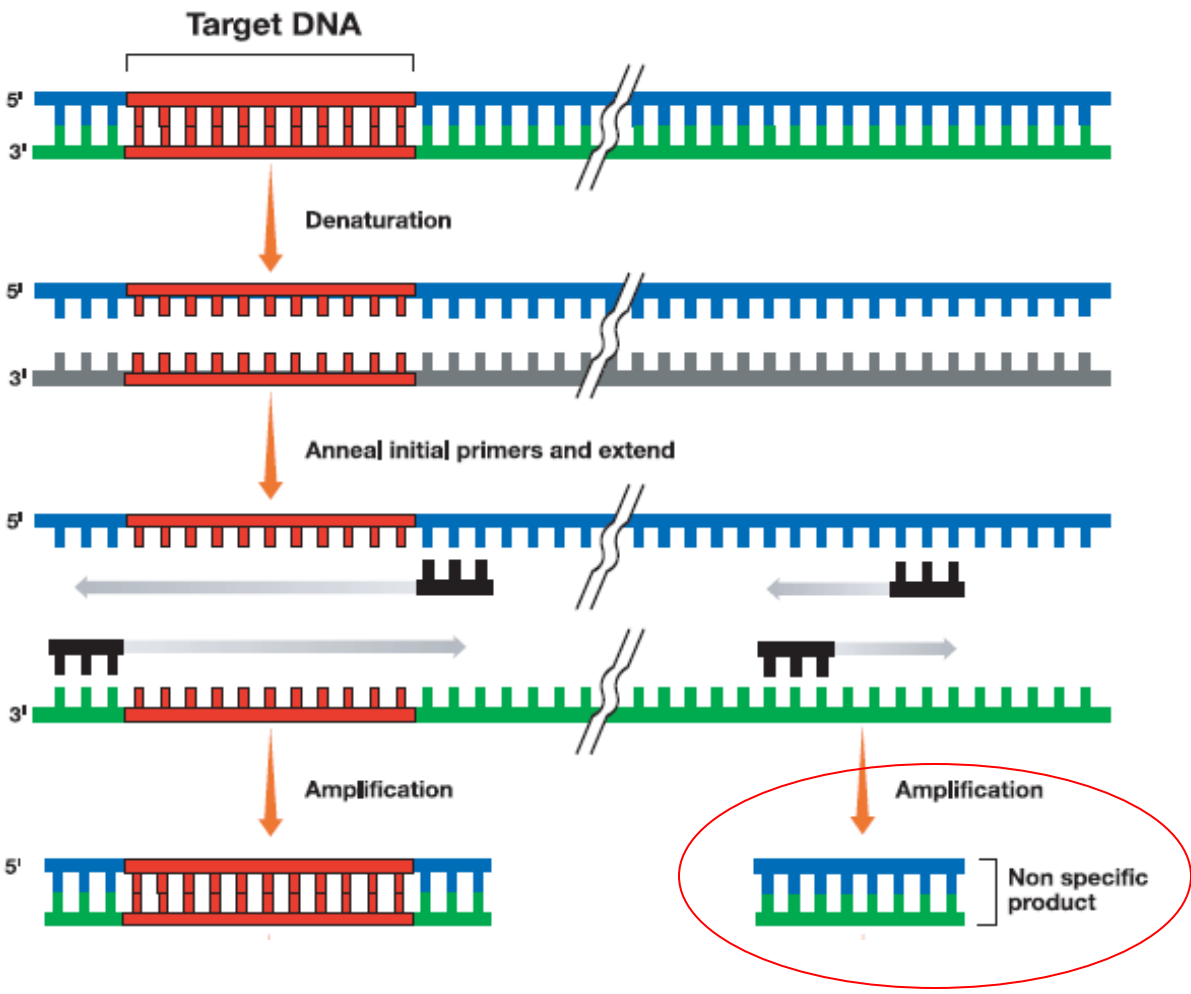
Lane 4- PCR of **lamb's ear gDNA** with initial primers (20 µl)

Lane 5- PCR of **pGAP plasmid control** with initial primers (20 µl)

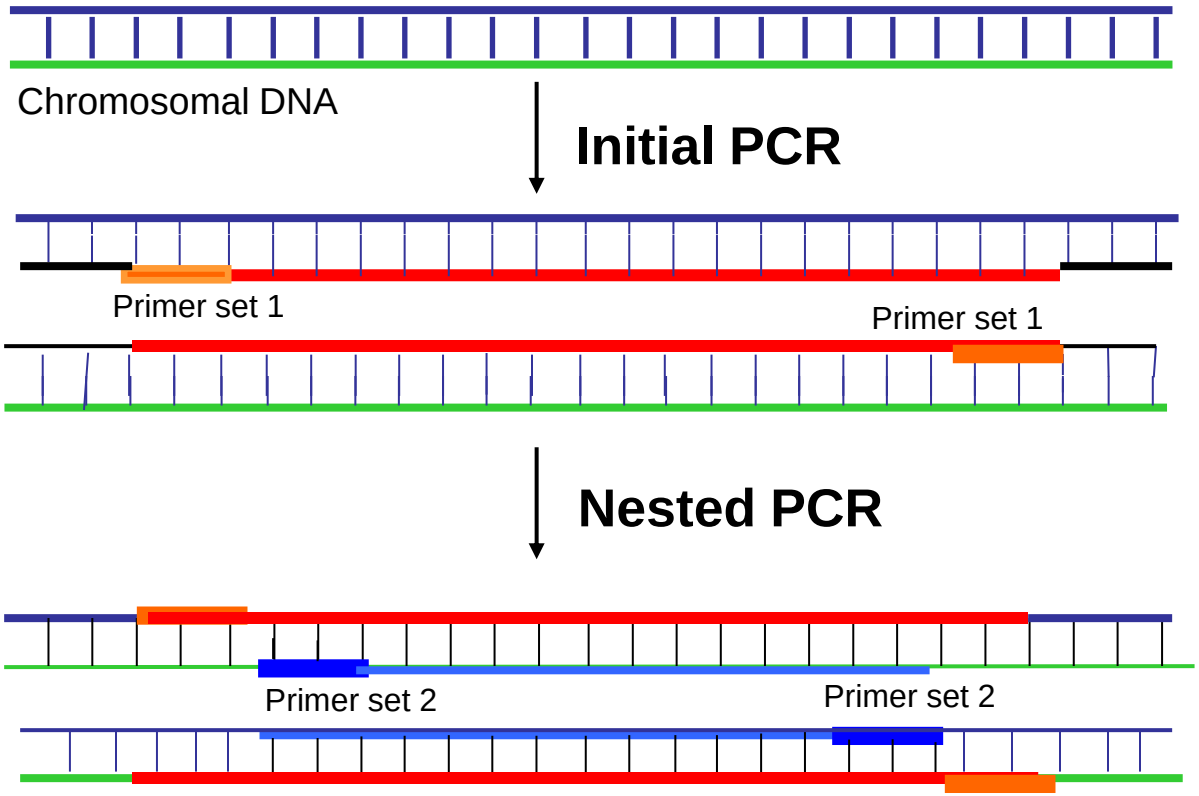
Sometimes no amplification is observed with initial PCR with some plants, or much fainter than this gel

Initial PCR may result in some amplicons that are non-specific

Because of the degenerate primer used to amplify the *GAPDH* of various plant species the initial PCR may also result in some non-specific amplifications



Nested PCR is used to amplify specifically within the amplicon (PCR product) of interest



Why is a Nested PCR reaction necessary?

- **Use of degenerate primers may not give you an exact match to the target sequence**

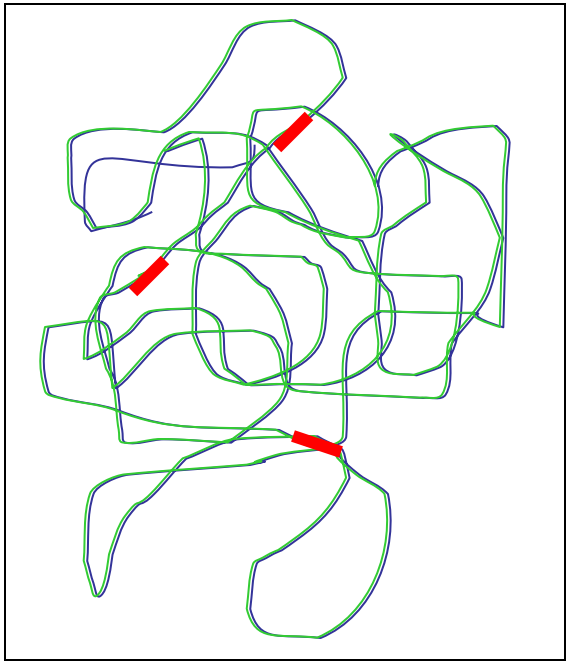
```
INITIAL                GACTATGTTGTTGAGTCTTCTGG
FORWARD PRIMER

Arabidopsis            GACTACGTTGTTGAGTCTACTGG
GAPC1AT3G04120
```

- **Because there are multiple primers in the mix, the primer concentration for the matching primer is lower than normal (1/12th concentration)**
- **Problems with initial PCR:**
  - inefficient
  - non-specific
- **Benefits of initial PCR:**
  - cast a wide net
  - increase the pool of specific products

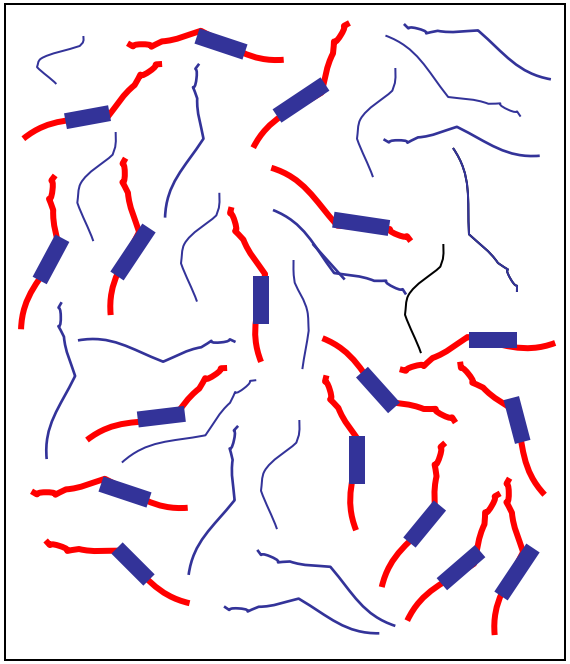
Using Nested PCR to increase your final PCR product

Initial PCR



DNA template: Genomic DNA

Nested PCR



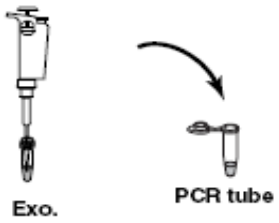
DNA template: Initial PCR products

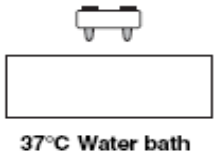
- **There is more PCR product from the nested PCR reactions since there is more specific template DNA to start from**
- **Results: intense, bold band on agarose gel**

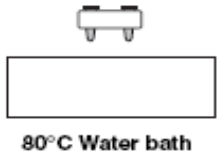
Exonuclease 1 treatment is needed before the second round of PCR (nested PCR) is done

- The primers that were not incorporated into PCR product in the first reaction must be removed so that they do not amplify target DNA in the second round of PCR.
- Exonuclease I will be added to the PCR products
- The enzyme must be inactivated before proceeding to the nested PCR

Exo treatment







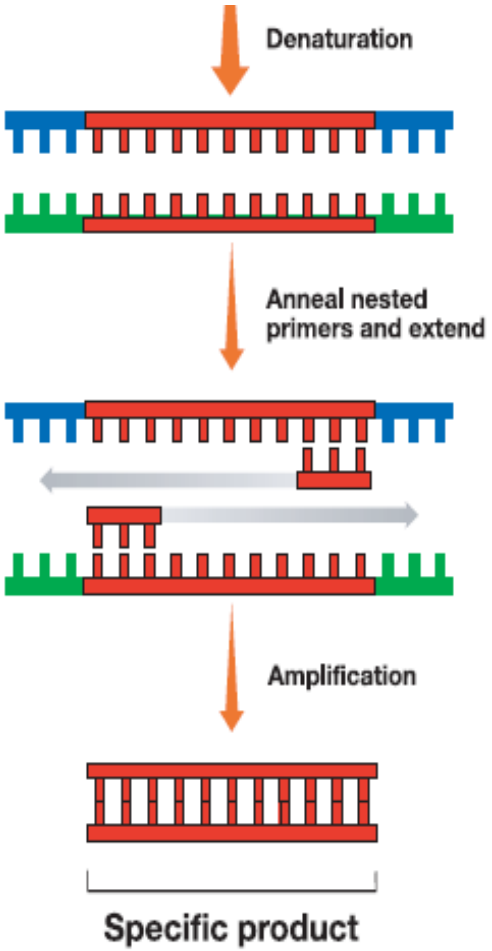
Protocol

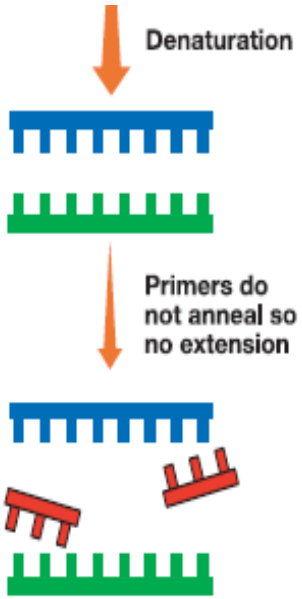
1. **Add 1 µl of exonuclease 1 enzyme to the PCR reactions**
2. **Incubate 15 min 37°C**
3. **Incubate 15 min 80°C**
4. **Dilute 2 µl Exo-treated PCR product in 98 µl water**

Note: Thermal cycler can be programmed for exo incubations

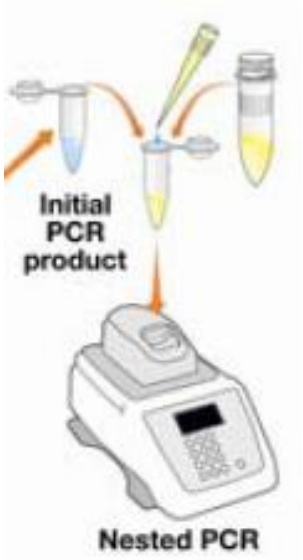
Nested PCR amplifies only regions within the *GAPDH* gene

Nested PCR is more specific





Setting up Nested PCR Reactions

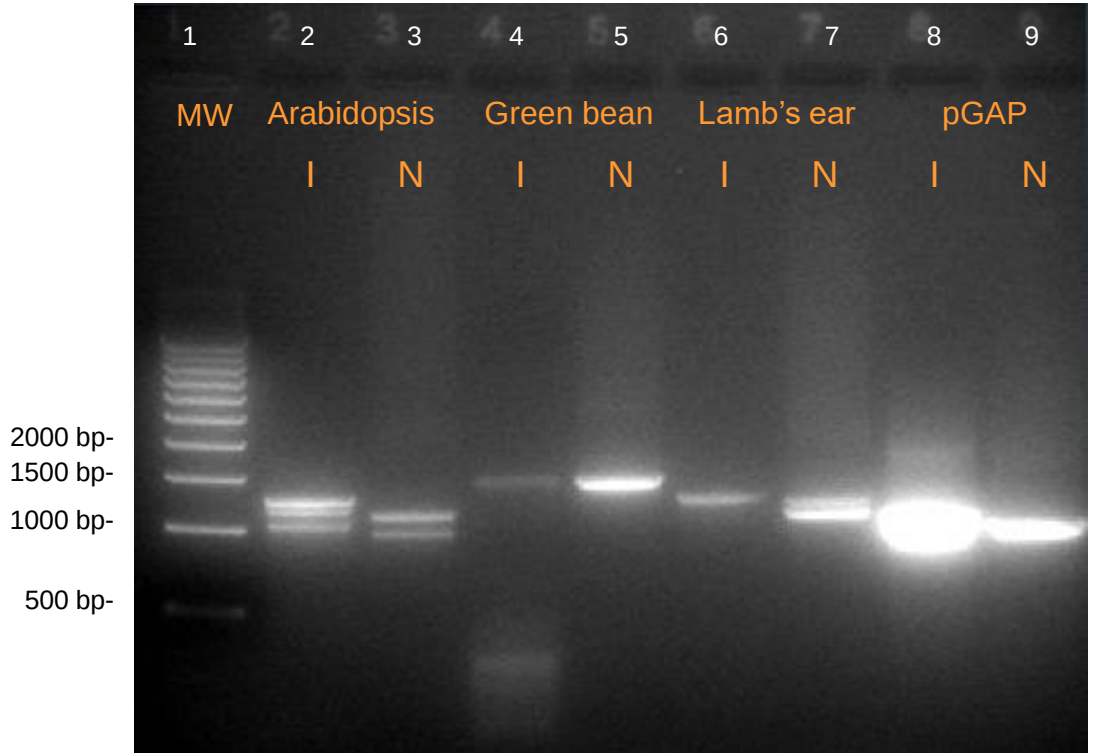


Protocol

1. **Mix 20 µl of diluted exo-treated template DNA with 20 µl of yellow mastermix with nested primers**
2. **For controls, mix 20 µl of control pGAP plasmid and 20 µl of water with 20 µl of yellow mastermix with nested primers**
3. **Amplify in thermal cycler (Annealing temp 46°C)**

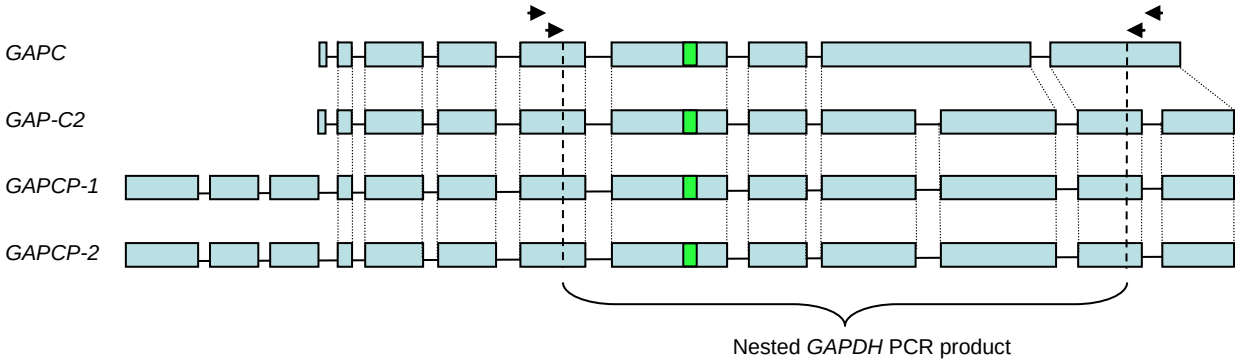
PCR results

1% agarose gel loaded with 20 µl initial PCR samples and 5 µl nested PCR samples.



PCR Products

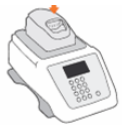
Initial vs. Nested Reactions



Arabidopsis *GAPC* Gene	Length of PCR product (bp)	
	Initial primers	Nested primers
GAPC	1065	993
GAPC-2	1216	1145
GAPCP-1	1303	1231
GAPCP-2	1205	1133

Genomic DNA Extraction
DNA Precipitation
DNA Quantitation

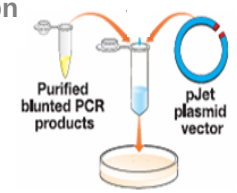
***GAPDH* PCR**
Nested PCR
Degenerate primers
Exonuclease



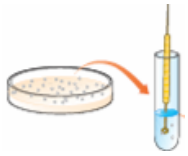
Gel Electrophoresis
DNA Gel Interpretation
Band Identification
Standard Curve Use

PCR Purification
Size Exclusion Chromatography

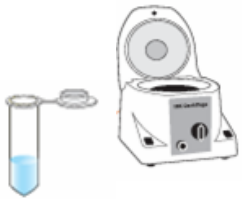
Cloning
Direct PCR cloning
Transformation
Ligation



Microbial Culturing
Antibiotic Selection
Sterile Technique

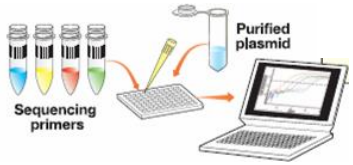


Plasmid Miniprep
Restriction Enzyme
Digestion
Gel Electrophoresis



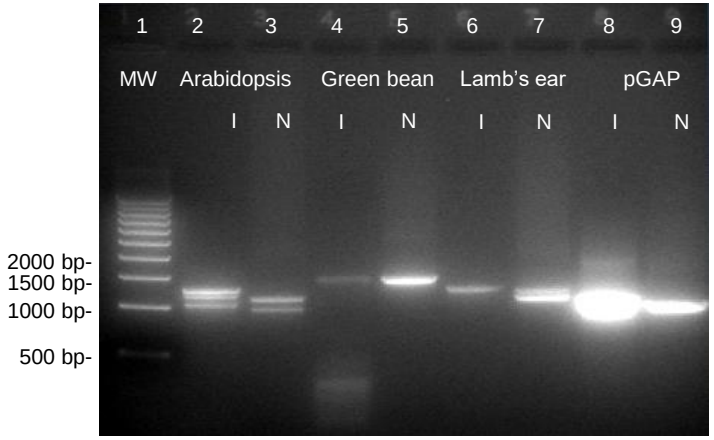
Sequencing
Automated sequencing

Bioinformatics
Sequence Data Editing
Contig Assembly
Intron-Exon Prediction



Cloning and Sequencing Explorer Series
GAPDH PCR Module

Results of Initial and Nested PCR



Lane 1- 500 bp molecular weight ruler (10 µl),
Lane 2- *Arabidopsis* gDNA with initial primers (20 µl)
Lane 3- Initial Arabidopsis PCR product with nested primers (5 µl)
Lane 4- Green bean gDNA with initial primers (20 µl)
Lane 5- Initial Green bean PCR product with nested primers (5 µl)
Lane 6- Lamb's ear gDNA with initial primers (20 µl)
Lane 7- Initial Lamb's ear PCR product with nested primers (5 µl)
Lane 8- pGAP plasmid control with initial primers (20 µl)
Lane 9- pGAP plasmid control with nested primers (5 µl)

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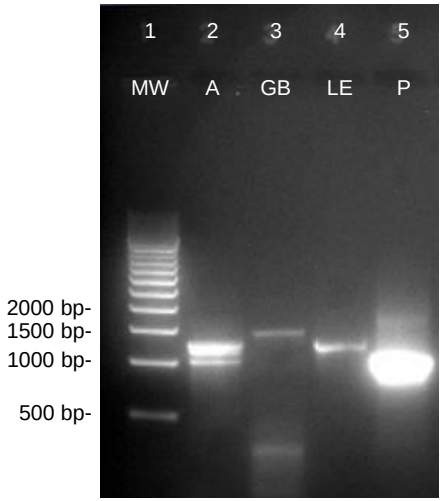
Cloning and Sequencing Explorer Series
GAPDH PCR Module

Results of Initial PCR



Lane 1- 500 bp molecular weight ruler (10 µl),
Lane 2- Control *Arabidopsis* gDNA with initial primers (20 µl)
Lane 3- Green bean gDNA with initial primers (20 µl)
Lane 4- Lamb's ear gDNA with initial primers (20 µl)
Lane 5- pGAP plasmid control with initial primers (20 µl)

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- **Real-Time PCR — What You Need To Know and Why You Should Teach It!**
- **Proteins — Where DNA Takes on Form and Function**
- **From plants to sequence: a six week college biology lab course**
- **From singleplex to multiplex: making the most out of your realtime experiments**

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