

- Polymerase Chain Reaction (PCR)
- DNA cloning strategies

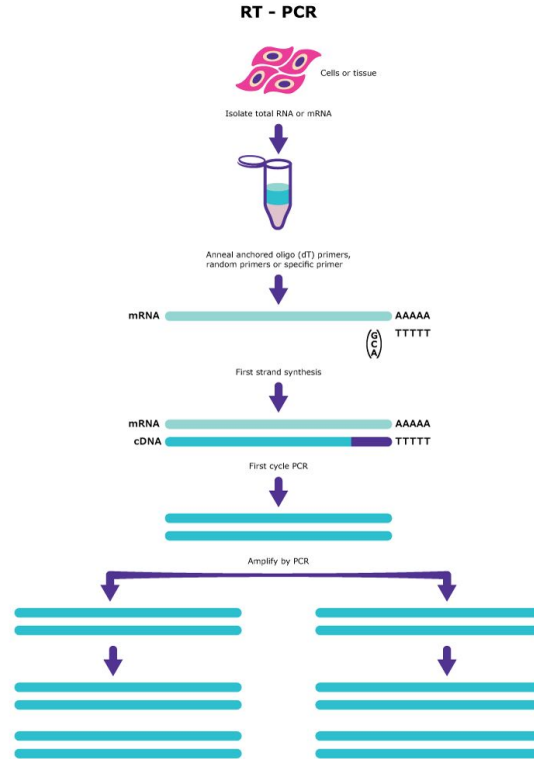
PCR amplification of Amy2 cDNA

- Objectives:
 - To amplify Amy2 coding sequence by PCR in order to clone it into a mammalian expression vector.
 - To analyze the PCR products (amplicons) by agarose gel electrophoresis.

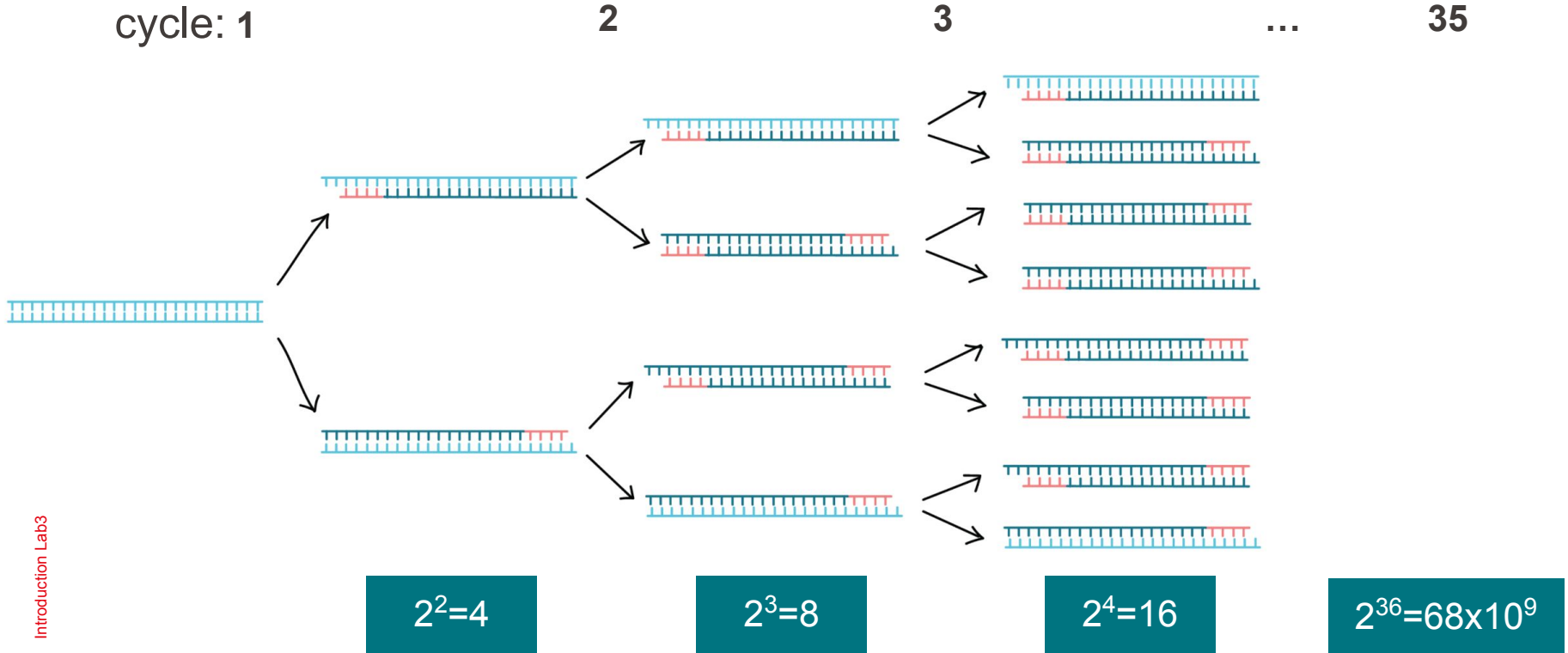
PCR template: pancreas-specific cDNA pool

laboratory 2

laboratory 3

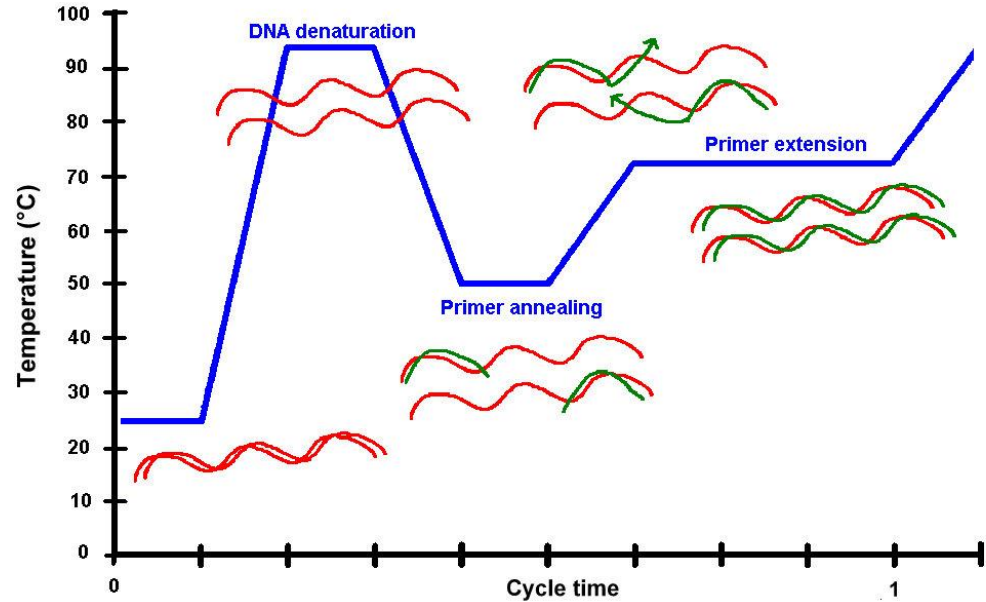


PCR amplification is exponential

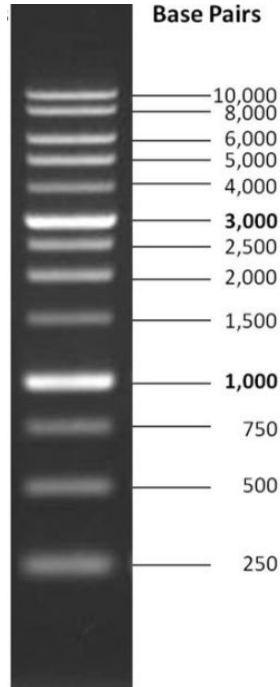


- A. **(+) RT sample:** cDNA produced by reverse transcription from mouse pancreatic total RNA
- B. **(-) RT negative control:** RT reaction lacking reverse transcriptase. Used to detect potential contamination of the RNA sample by DNA.
- C. **PCR positive control:** plasmid DNA containing the sequence of interest. PCR product of condition A should show identical profile to that of C.
- D. **PCR negative control:** water instead of template to detect potential contamination of reagents. No amplification should be observed.

- Three cycling steps:
 - Denaturation (95 °C)
 - Annealing (~45-60 °C)
 - Extension (72 °C)

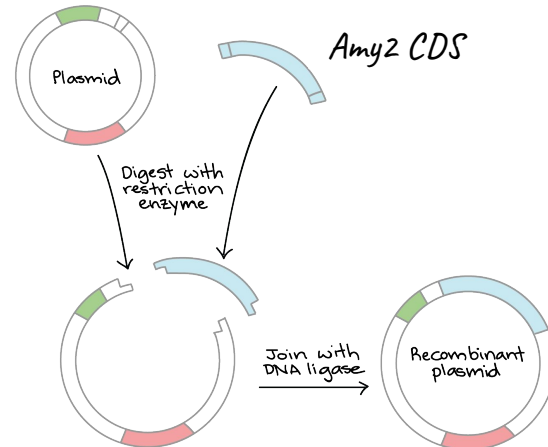


Analysis of PCR products by agarose gel electrophoresis



- **DNA ladder** consist of several DNA fragments with different length
- You can estimate the size of the amplified **PCR product** by comparison to the ladder
- A specific dye (**GelRed**) in the agarose gel **intercalates** between DNA base pairs
- Fluorescent dye-nucleic acid complexes are then visualized with **UV light**

- Objectives:
 - To retrieve a sequence of interest from NCBI database.
 - To design cloning strategies using Benchling software.



Retrieve sequence of interest in NCBI database

NCBI Resources How To

Nucleotide Nucleotide

Limits Advanced

Display Settings: GenBank

Mus musculus amylase 2a5 (Amy2a5), mRNA

NCBI Reference Sequence: NM_001042711.2

FASTA Graphics

Go to:

LOCUS NM_001042711 1577 bp mRNA linear ROD 15-AUG-2011

DEFINITION Mus musculus amylase 2a5 (Amy2a5), mRNA.

ACCESSION NM_001042711 NM_001042712 NM_009669

VERSION NM_001042711.2 GI:142356897

KEYWORDS .

SOURCE Mus musculus (house mouse)

ORGANISM [Mus musculus](#)
Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
Mammalia; Eutheria; Euarchontoglires; Glires; Rodentia;
Sciurognathi; Muroidea; Muridae; Murinae; Mus; Mus.

REFERENCE 1 (bases 1 to 1577)

Scroll down

Find Amy2 coding sequence (CDS)

```

CDS      18..1544
/gene="Amy2a5"
/gene_synonym="1810008N23Rik; Amy-2; Amy2; mAmy2-2"
/EC_number="3.2.1.1"
/note="amylase 2a5, pancreatic; pancreatic alpha-amylase;
PA; 1,4-alpha-D-glucan glucanohydrolase; amylase 2,
pancreatic"
/codon_start=1
/product="pancreatic alpha-amylase precursor"
/protein_id="NP_001036176.1"
/db_xref="CCDS:CCDS17775.1"
/db_xref="GeneID:109959"
/db_xref="MGI:MGI:88020"
/translation="MKFVLLLSLIGFCWAQYDPHTSDGRTAIVHLFEWRWVDIAKECE
RYLAPKGFGGVQVSPNENVVHNPSPRPWERYQPISYKICTRSGNEDEFDMVTRCN
NVGVRIYVDVINHMCAGNPAGTSSTCGSYLNPNNREFPAVPYSAWDFNDNKCNGEI
DNYNDAYQVRNCRLTGLLDLALEKDYVRTKVADYMNHLIDIGVAGFRDLAAKHMWPGD
IKAVLDKHLNLTWKFSQGSRPPIFQEVIDLGGEAIKGSEYFGNGRVTEFKYGAKLGT
VIRKWNGEKMSYLNWGEWGLVPSDRALVFVDNHDNQRGHGAGGSSILTFWDARMYK
MAVGFMLAHPYGFTRVMSSYRWNRNFQNGKDQNDWIGPPNNGVTKEVTINADTTCGN
DWVCEHRWRQIRNMVAFRNVVNGQFFSNWWDNNSNQVAFSRGNRGFIVFNDDWALSA
TLQTGLPAGTYCDVISGDKVDGNCTGLRVNVGSDGKAHFSISNSAEDPFIHADS KL
"

sig_peptide 18..62
/gene="Amy2a5"

```

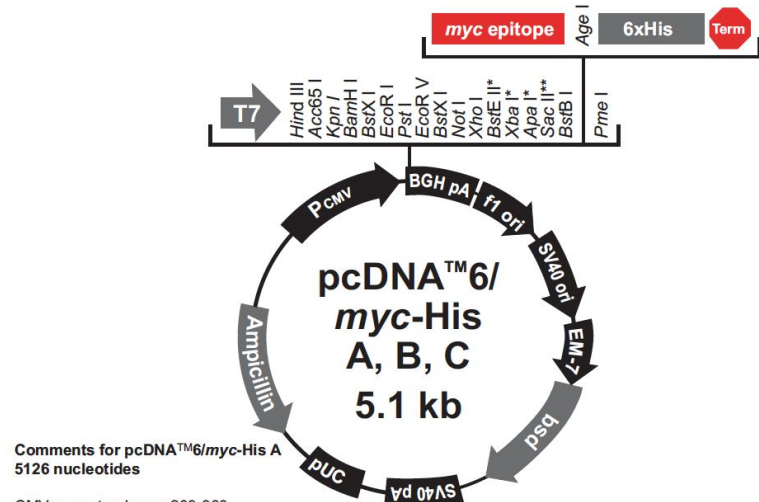
Find the start and stop codon within

```

1  gacaacttca aagcaaaatg aagttcggtc tgctgcttcc cctcattggg ttctgctggg
61 ctcaatatga cccacatact tcagatggga ggactgctat tgtccacctg ttcgagtggc
121 gctgggttga tattgccaaag gaatgtgagc gatacttagc tcctaaggga ttgggagggg
181 tgcagggtctc tccaccaaat gaaaacgttg tagttcataa cccatcaaga ccttgggtggg
241 aaagatacca accaatcagc tataaaatct gcacaaggtc tggaaatgaa gatgaattca
301 gagacatggt gacaagggtc aacaatgttg gtgtccgtat ttatgtggat gctgtcatta
361 accacatgtg tggcgcaggg aatcctgcag gaacaagcag tacctgtgga agttacctca
421 atccaaataa caggggaattc ccagcagttc catactctgc ttgggaacttt aacgataata
481 aatgtaatgg agaaattgat aactacaatg atgcttatca ggtcagaaat tgcgtctctga
541 ctggccttct ggatcttgca cttgagaaag attatgttcg taccaagggtg gctgactata
601 tgaacctctc cattgacatt ggagtagcag ggttcagact tgatgctgct aagcacatgt
661 ggcttgagga cataaaggca gttttggaca aactgcataa tctcaataca aaatggttct
721 ccaaggaag cagaccttct attttccaag aggtcattga tctgggtggt gaggcaatta
781 aaggtagtga gtactttgga aatggccgtg tgacagaatt caagtatggt gcaaaacttg
841 gcacagttat ccgcaagttg aatggcgaga agatgtccta tttaaagaac tggggagaag
901 gttgggttct ggtgccttct gacagagccc ttgtgtttgt ggacaaccat gataatcagc
961 gaggacatgg tgcgtgagga tcatccatcc tgacattctg ggatgctaga atgtataaaa
1021 tggctgtcgg atttatgttg gctcatcctt atggattcac aagagtaatg tcaagttacc
1081 gttggaatag aaatttccag aatggaaaag atcagaatga ctggattgga ccaccaataa
1141 acaatggagt acaaaaagaa gtgaccatta atgcagacac tacttgtggc aatgactggg
1201 tctgtgaaca cagatggcgt caaatcagga acatggttgc cttcaggaat gtggtcaatg
1261 gtcagccttt ttcaactcgt tgggataata acagcaacca agtagctttt agcagaggga
1321 acagaggatt cattgtcttt aacaatgatg actgggcttt gtcagccact ttacagactg
1381 gtcttctctg tggcacatac tgtgatgtca tctctggaga taaggtcgat ggcaattgca
1441 ctggacttag agtgaatggt ggcagtgatg gcaaagctca cttttccatt agtaactctg
1501 ctgaggaccc atttattgca atccatgctg actcaaaatt gtaaatct atattaaaga
1561 gatttggatt aagcatc

```

Cloning of Amy2 CDS in mammalian expression plasmid



CMV promoter: bases 209-863
 T7 promoter/priming site: bases 863-882
 Multiple cloning site: bases 902-999
 myc epitope: bases 997-1026
 Polyhistidine tag: bases 1042-1059
 BGH reverse priming site: bases 1082-1099
 BGH polyadenylation signal: bases 1081-1295
 f1 origin: bases 1358-1771
 SV40 promoter and origin: bases 1813-2121
 EM-7 promoter: bases 2169-2224
 Blastidicin resistance gene: bases 2249-2641
 SV40 polyadenylation signal: bases 2799-2929
 pUC origin: bases 3312-3985
 Ampicillin resistance gene: bases 4130-4991

*There is a unique BstE II site, but no Xba I or Apa I sites in version C.

There is a unique Sac II site between the Apa I site and the BstB I site in **version B only.

Primer design for protein expression

- Cloning of Amy2 coding sequence into expression plasmid:
 - The forward primer includes the start codon.
 - For C-terminal fusion remove stop codon.
 - Add suitable restriction sites at the 5' end of your primers and 2-3 additional bases at the 5' end (required for optimal cleavage by the restriction enzyme).
- Protein production in eukaryotic cells:
 - Add Kozak consensus sequence (ACCATGG) if not present on plasmid.
 - This regulatory sequence allows efficient translation initiation in eukaryotic cells.
 - Check the reading frame!

Check the open reading frame (ORF) of Amy2 fusion protein

- The ORF includes the Amy2 start codon, Amy2 CDS, myc-HIS tag and ends after the stop codon (in the plasmid)
- The plasmid is provided with three different reading frames A, B and C.
- Primer and plasmid must be chosen to create the correct fusion protein
- The addition or deletion of a base pair(s) in the primers may cause a frameshift, resulting in the translation of the genetic code in an unnatural reading frame

Frameshift mutation

