Overview of Lecture Series

1. Determining the molecular identity of receptors

Getting the first clone

- Classical approach
- Expression cloning

Isoforms & Evolutionary Precursors

- PCR

2. Determining the stoichiometry of receptors

Selective Tagging

Constraining Stoichiometry

3. Determining the structure of receptors

Membrane Topology

- Hydropathy Plots
- Engineered Reporter Sites

3-Dimensional Structure

- Electron Cryomicrocopy
- X-ray crystallography

Methodological Approaches To Study Receptors

Lecture #1

Determining The Molecular Identity of Receptors

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Determining the Molecular Identity of Receptors

Overview

Phase 1: *Getting The First Clone*

* Classical Approach e.g. nAChRs
  Positional Cloning e.g. K-channels
* Expression Cloning e.g. iGluRs

Phase 2: *Isoforms & Evolutionary Precursors*

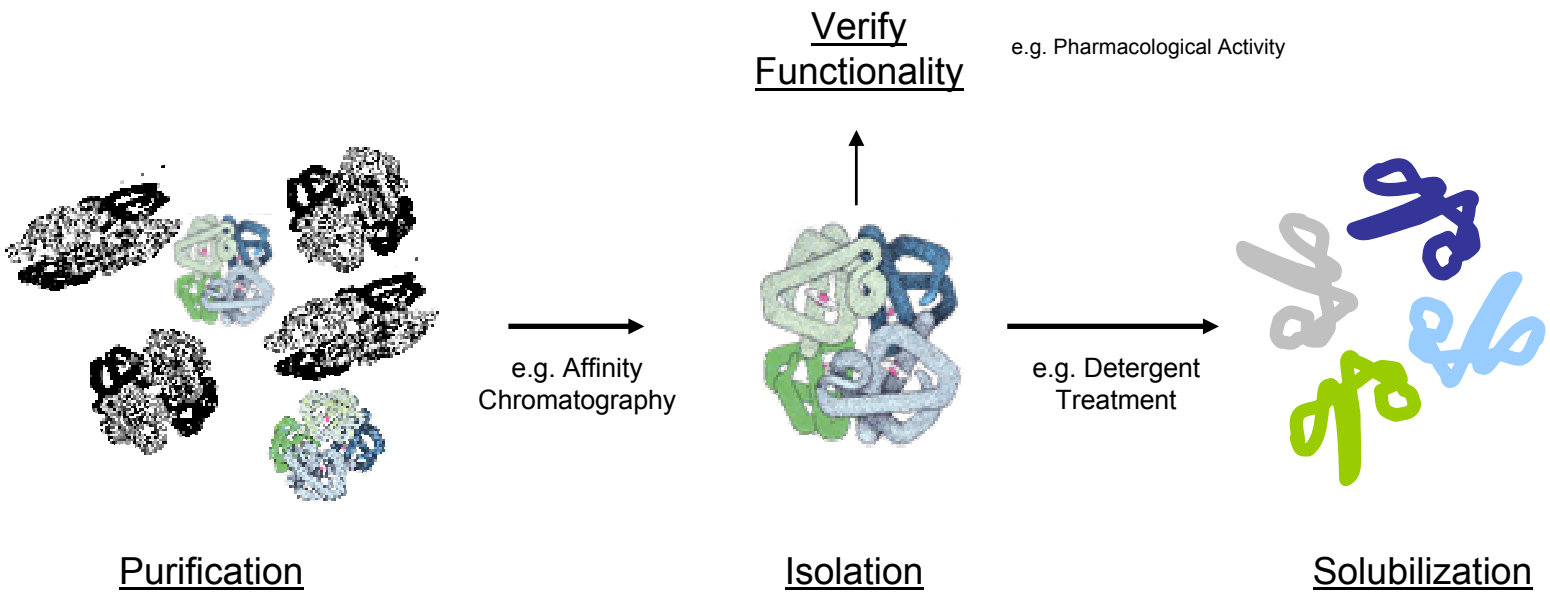
* Low-Stringency Hybridization
  Polymerase Chain Reaction
  Searching Databases

Determining the Molecular Identity of Receptors

Getting the First Clone: Classical Approach

Step 1: Purify From A Source Rich In Protein of Interest

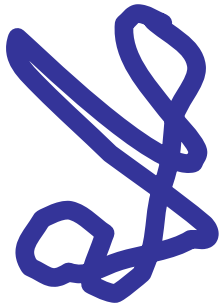
e.g. Na channel or nAChR: Electric organ of electric eel



Determining the Molecular Identity of Receptors

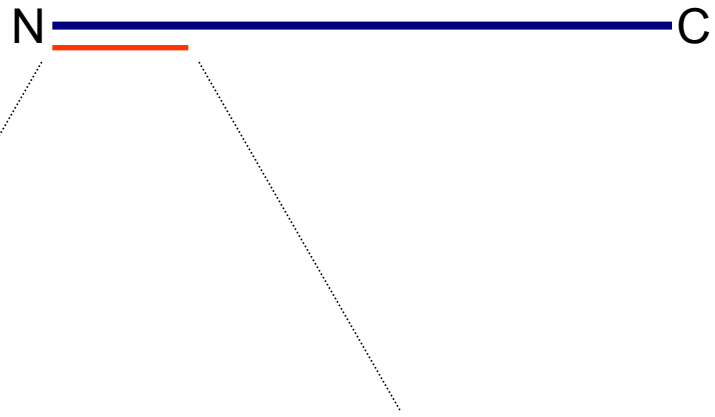
Getting the First Clone: Classical Approach

Step 2: Obtain Partial Sequence Of Protein



Individual Subunit

(e.g. Chemical sequencing of N-terminal AAs
Approx. first 50 residues)



SEHETRLVANLLENYNKVIRPVEHHTHFVDITVGLQLIQLISVDEVNQIV

e.g. α-subunit of nAChR, *Torpedo californica*

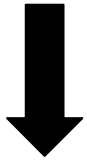
Determining the Molecular Identity of Receptors

Getting the First Clone: Classical Approach

Step 3: Synthesis Of Oligonucleotide Probes

Partial Protein Sequence

SEHETRLVANLLENYNKVIRPVEHHTHFVDITVGLQLIQLISVDEVNQIV



e.g. α-subunit of nAChR, *Torpedo californica*

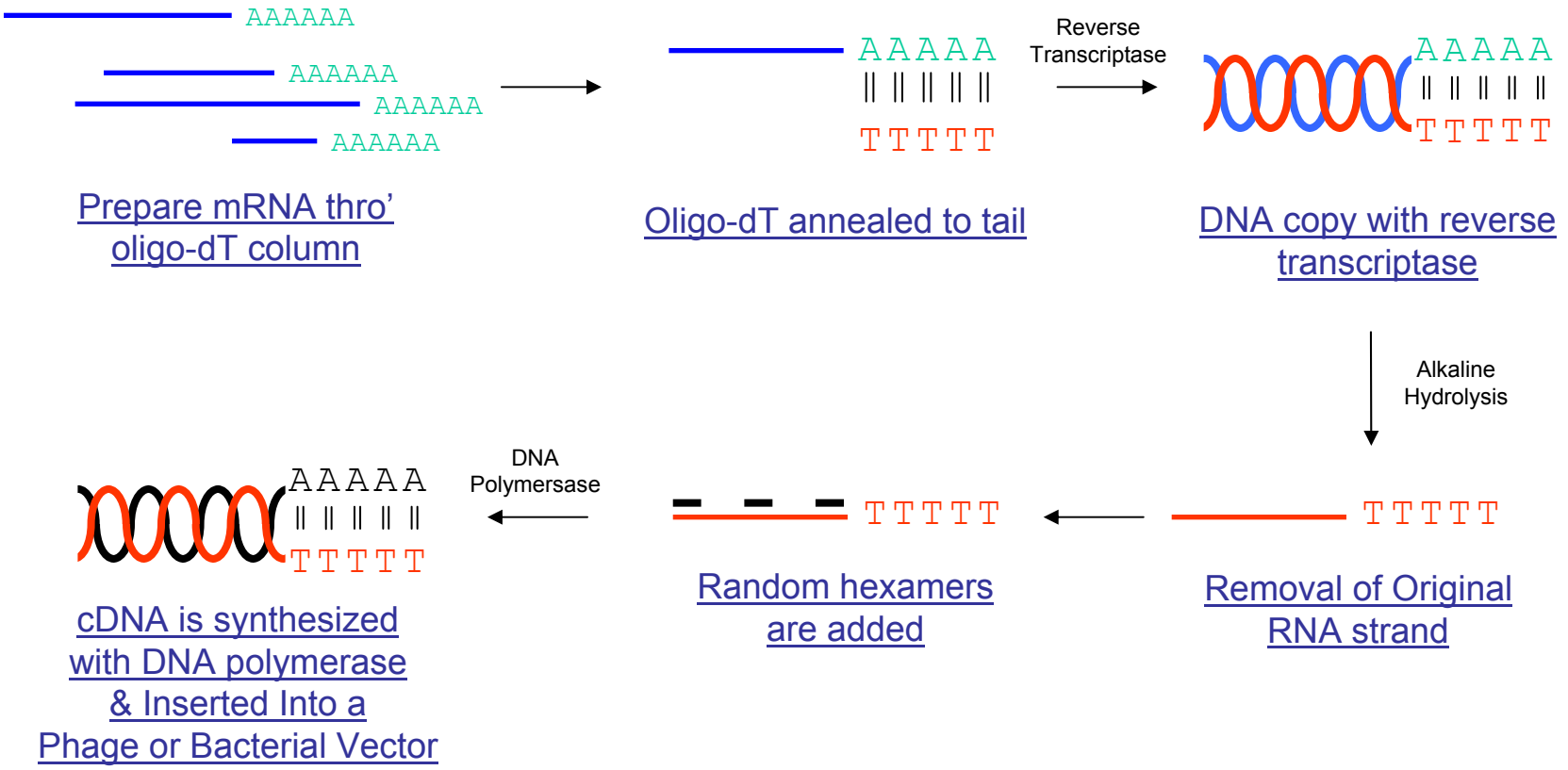
Nucleotide Sequence To Generate Probes For cDNA Library

ATCTCTTCACTAGAAAAGAGCTGAACACAGAAGTCCAGAAGATCTAACAAGTTCATCGTTTAGTTATTAGAAGTG
GCAGATTTGCTTGAAAAGCCAATTATTGAAAGCTGAAGAATGATTCTGTGCAGTTATTGGCATGTAGGGTTGGTG

Determining the Molecular Identity of Receptors

Getting the First Clone: Classical Approach

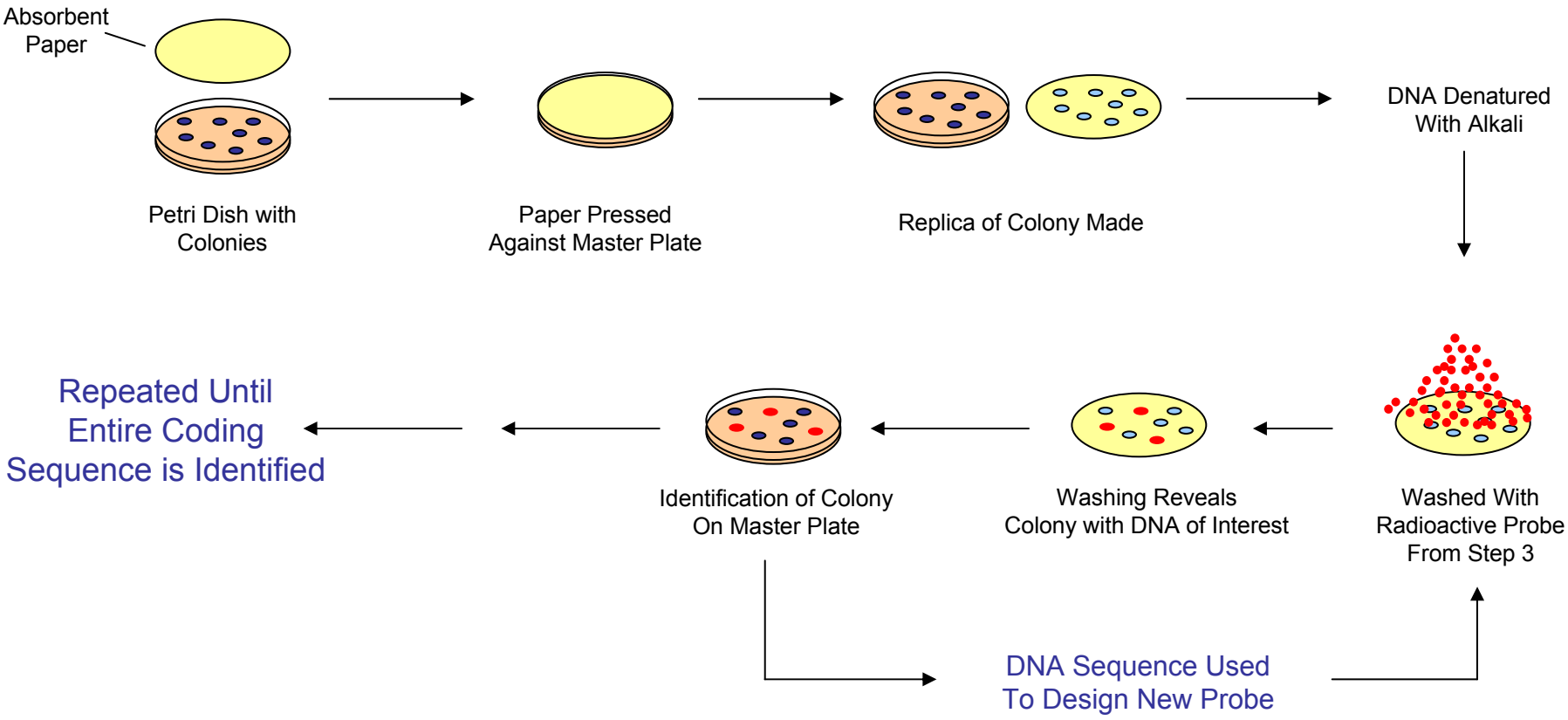
Step 4: Generating a cDNA Library



Determining the Molecular Identity of Receptors

Getting the First Clone: Classical Approach

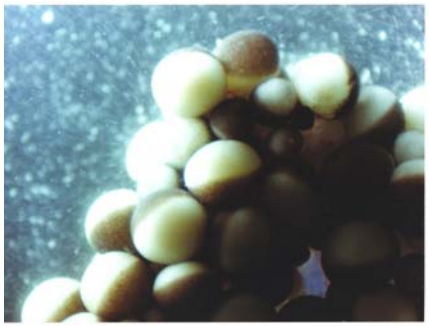
Step 5: Screening cDNA Library



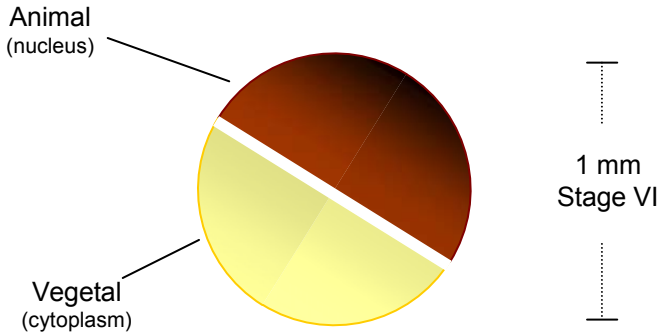
Determining the Molecular Identity of Receptors

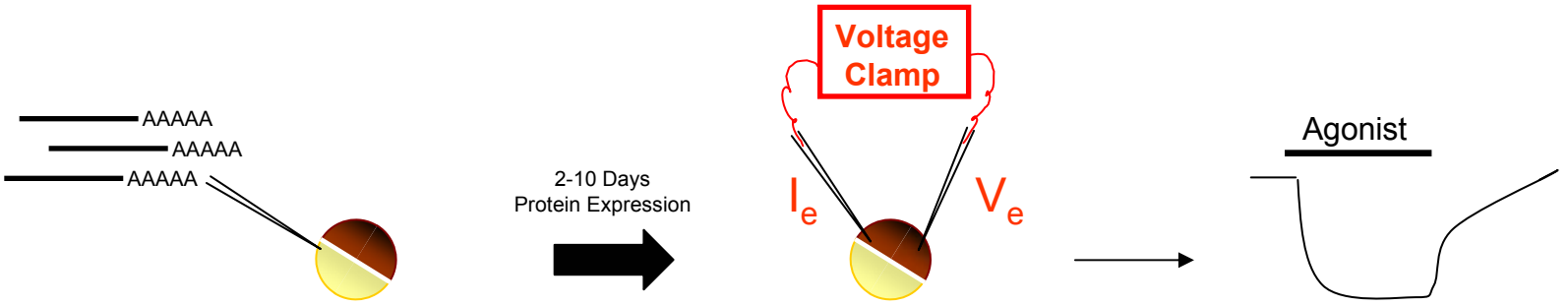
Getting the First Clone: Classical Approach

Step 6: Verify Function In Heterologous Expression System



Xenopus laevis oocytes





Inject With mRNA

Record Agonist Response

Agonist Evoked Membrane Current

Determining the Molecular Identity of Receptors

Getting the First Clone: Classical Approach

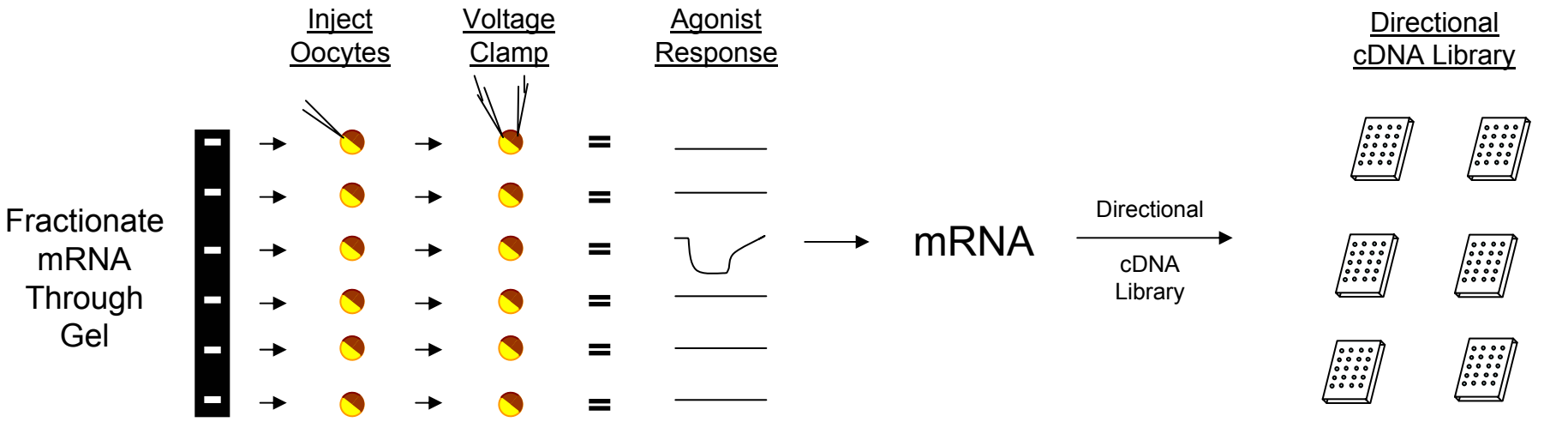
Benefits & Disadvantages

- Slow, requires protein chemistry, multi-disciplinary
- Limited, Needs Source Rich In Protein
- Successfully employed however e.g. nAChRs, $GABA_A$ & Glycine, Na channels

Determining the Molecular Identity of Receptors

Getting the First Clone: Expression Cloning

Step 1: Construct cDNA Library Enriched For Receptor Activity



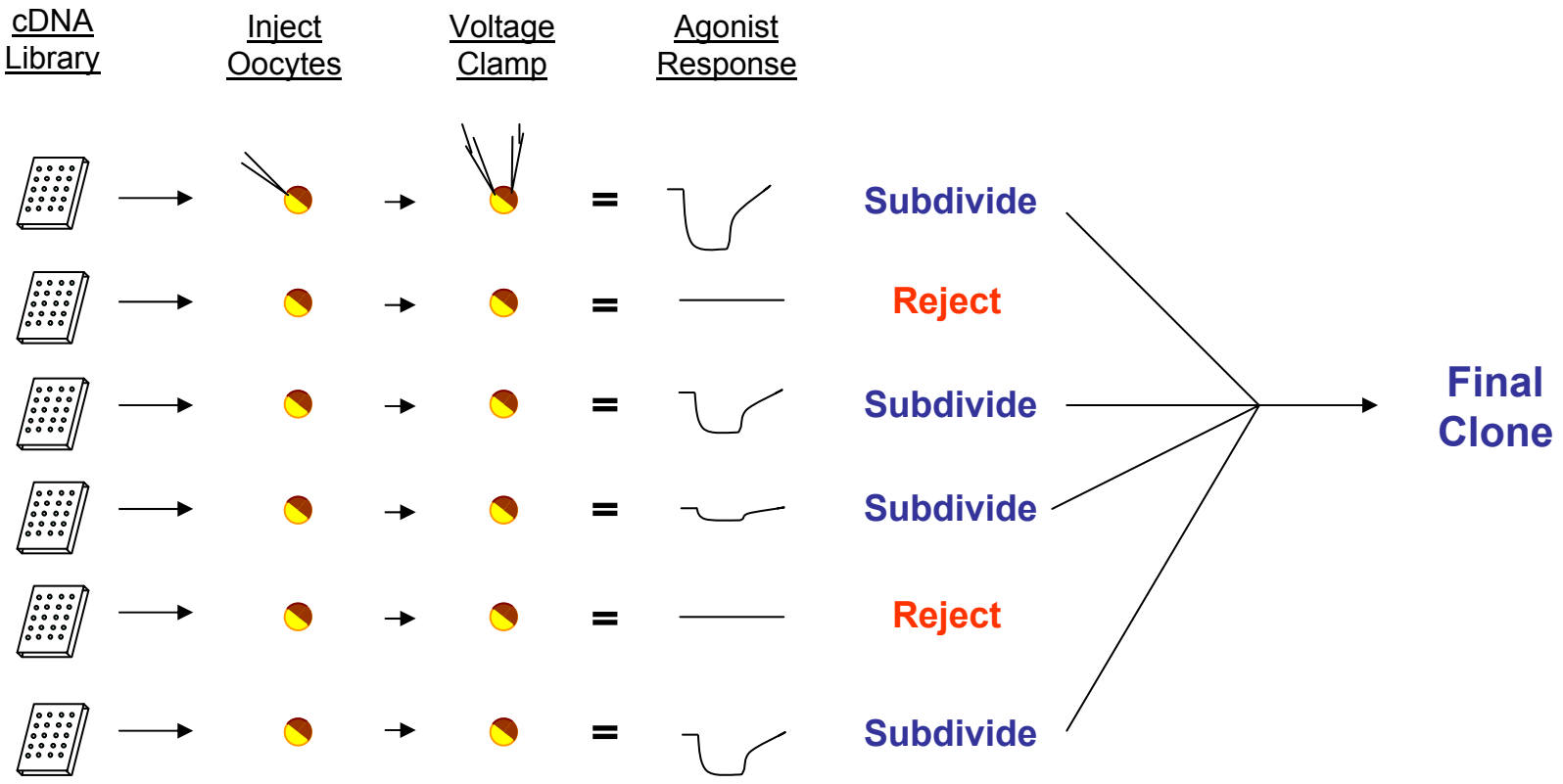
e.g. Source of Rat Forebrain, First iGluR clone
Hollmann *et al* (1989) Nature 342, 643-648

Consisting of Several Independent Sub-Libraries

Determining the Molecular Identity of Receptors

Getting the First Clone: Expression Cloning

Step 2: Screening of Sub-Libraries



Hollmann *et al* (1989) Nature 342, 643-648

Determining the Molecular Identity of Receptors

Getting the First Clone: Expression Cloning

Benefits & Disadvantages

- Faster, neither protein chemistry nor sequence info required
- Works if single mRNA makes a functional product
- Clone may represent an enzyme or protein that aids expression of receptor

Determining The Molecular Identity of Receptors

What Have We Learned So Far ?

1. Getting the first clone

Classical approach
Expression cloning

What's Next ?

2. Isoforms & Evolutionary Precursors

Polymerase Chain Reaction

Determining the Molecular Identity of Receptors

Isoforms & Evolutionary Precursors: PCR

Objective: Amplify Many Copies Of Homologous Sequences

Step 1: Setting Up Reaction Conditions

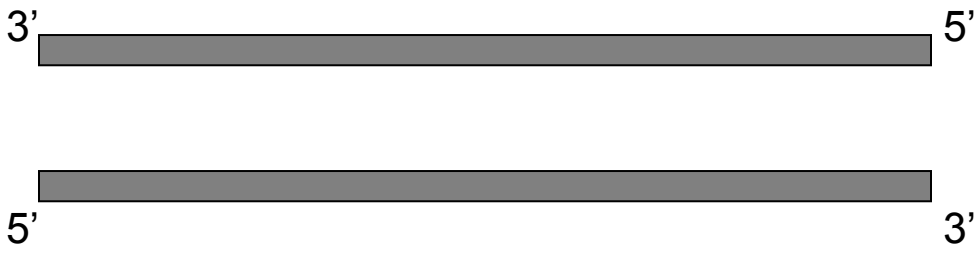
- Requires Sequence Information Of 2 Regions of DNA
- Two Primers Are Synthesized (15-20 nucleotides)
- Primers Are 200-2000 bps apart allowing 3' ends to point to each other

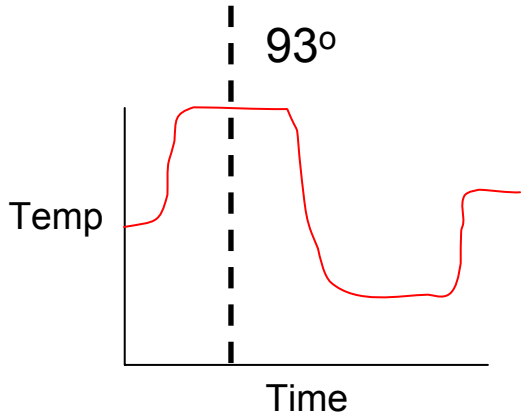
Determining the Molecular Identity of Receptors

Isoforms & Evolutionary Precursors: PCR

Step 2: Denaturation of Target DNA

High temperature dissociates target DNA into two strands



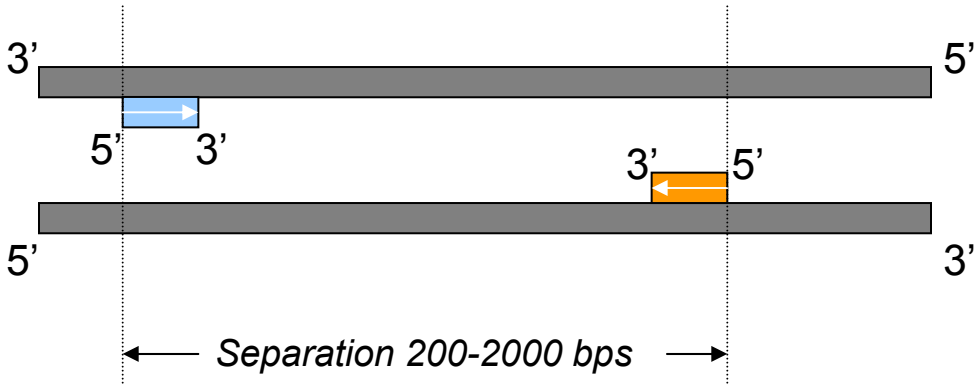


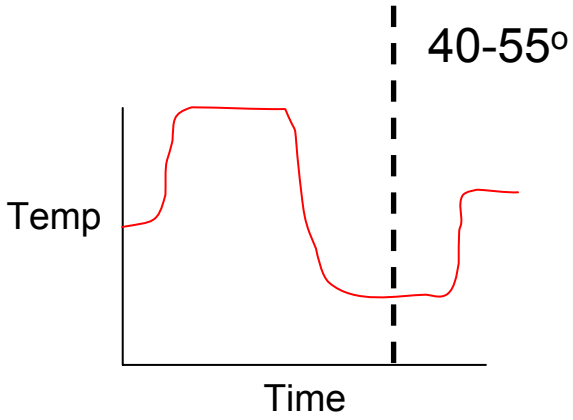
Determining the Molecular Identity of Receptors

Isoforms & Evolutionary Precursors: PCR

Step 3: Annealing Primers To Target DNA

Lower temperature permits primers to anneal

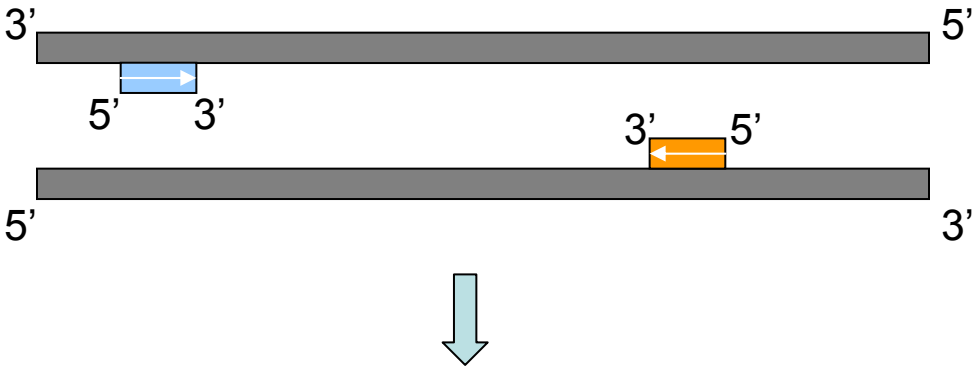




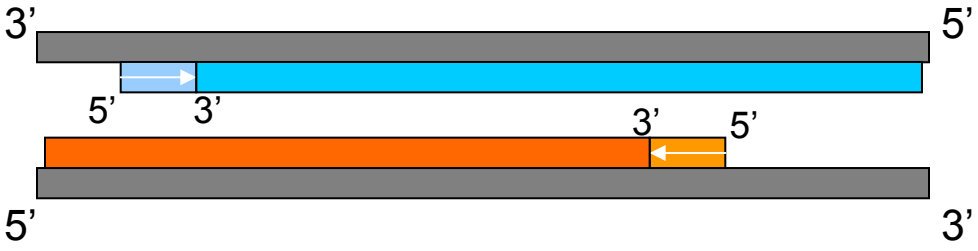
Determining the Molecular Identity of Receptors

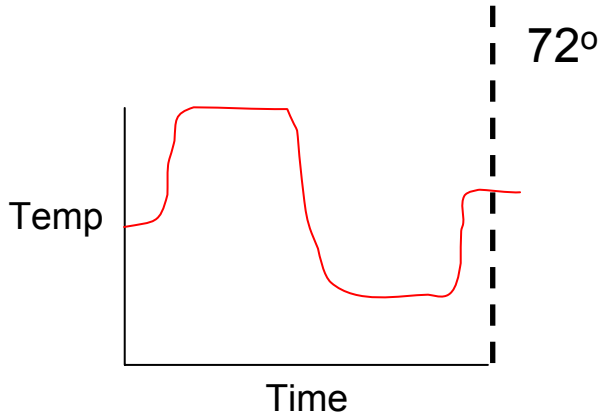
Isoforms & Evolutionary Precursors: PCR

Step 4: Elongation of DNA by DNA Polymerase



Increased temperature facilitates elongation by DNA polymerase

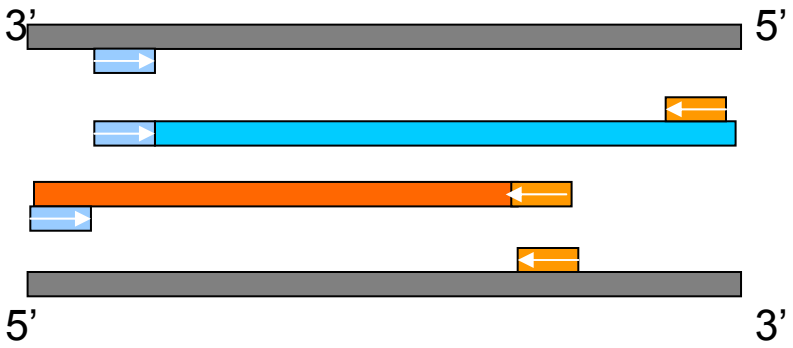


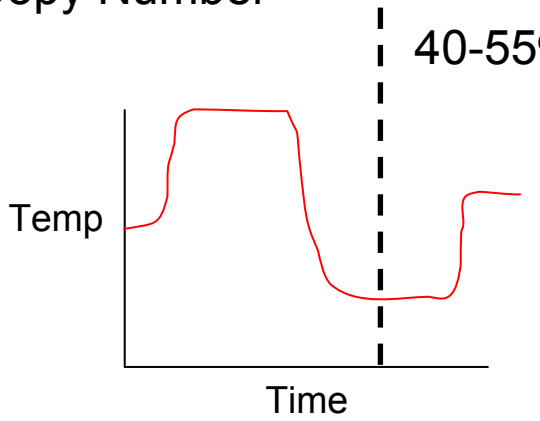


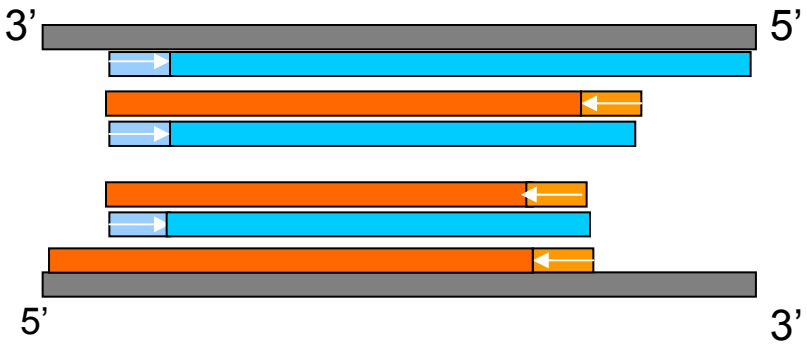
Determining the Molecular Identity of Receptors

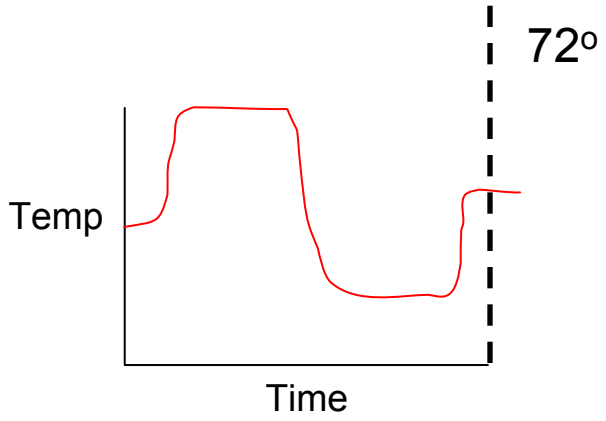
Isoforms & Evolutionary Precursors: PCR

Step 5: Second PCR Cycle Further Amplifies Copy Number





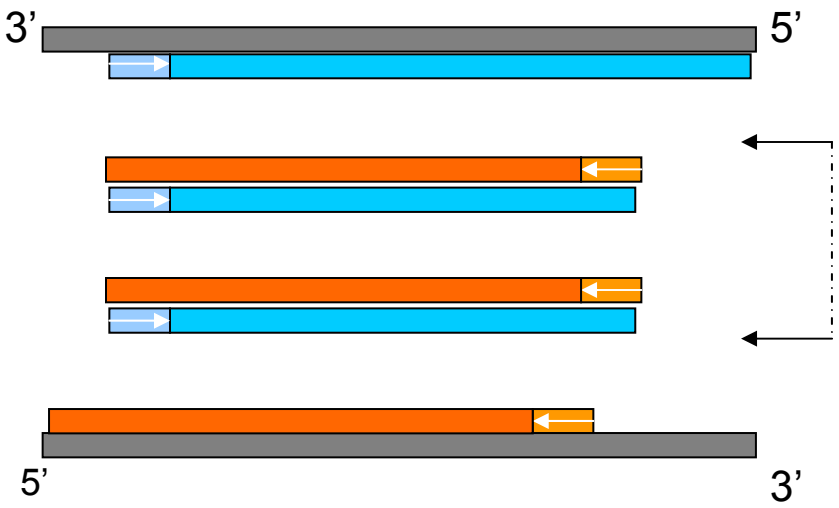




Determining the Molecular Identity of Receptors

Isoforms & Evolutionary Precursors: PCR

Step 6 onwards: Process is Repeated Many Times



Amplicons

After 30 cycles there will be 2^{30} copies
> 100 hundred million copies

Determining The Molecular Identity of Receptors

What Have We Learned?

1. Getting the first clone

   Classical approach
   Expression cloning

2. Isoforms & Evolutionary Precursors

   Polymerase Chain Reaction

Determining The Molecular Identity of Receptors

Further Reading

1. Snutch, T.P. (1988) The use of Xenopus oocytes to probe synaptic communication. TiNS, 11, 250-256.

2. Hille, B. (1996) Ion channels of excitable membranes; Structure of channel proteins Chapter 13, 3rd Edition, Sinauer Associates, Inc.

3. Simonsen, H. & Lodish, H.F. (1994) Cloning by function: expression cloning in mammalian cells. TiPS, 15, 437-441.

4. Hollmann *et al* (1989) Cloning by functional expression of a member of the glutamate receptor family. Nature, 342, 643-648.