Fundamentos de Biologia Molecular

Curso de Licenciatura em Biologia
2º Ano, 1º Semestre
Ano Letivo 2018/2019

Componente Teórico-Prática

Ciências ULisboa
Faculdade de Ciências da Universidade de Lisboa

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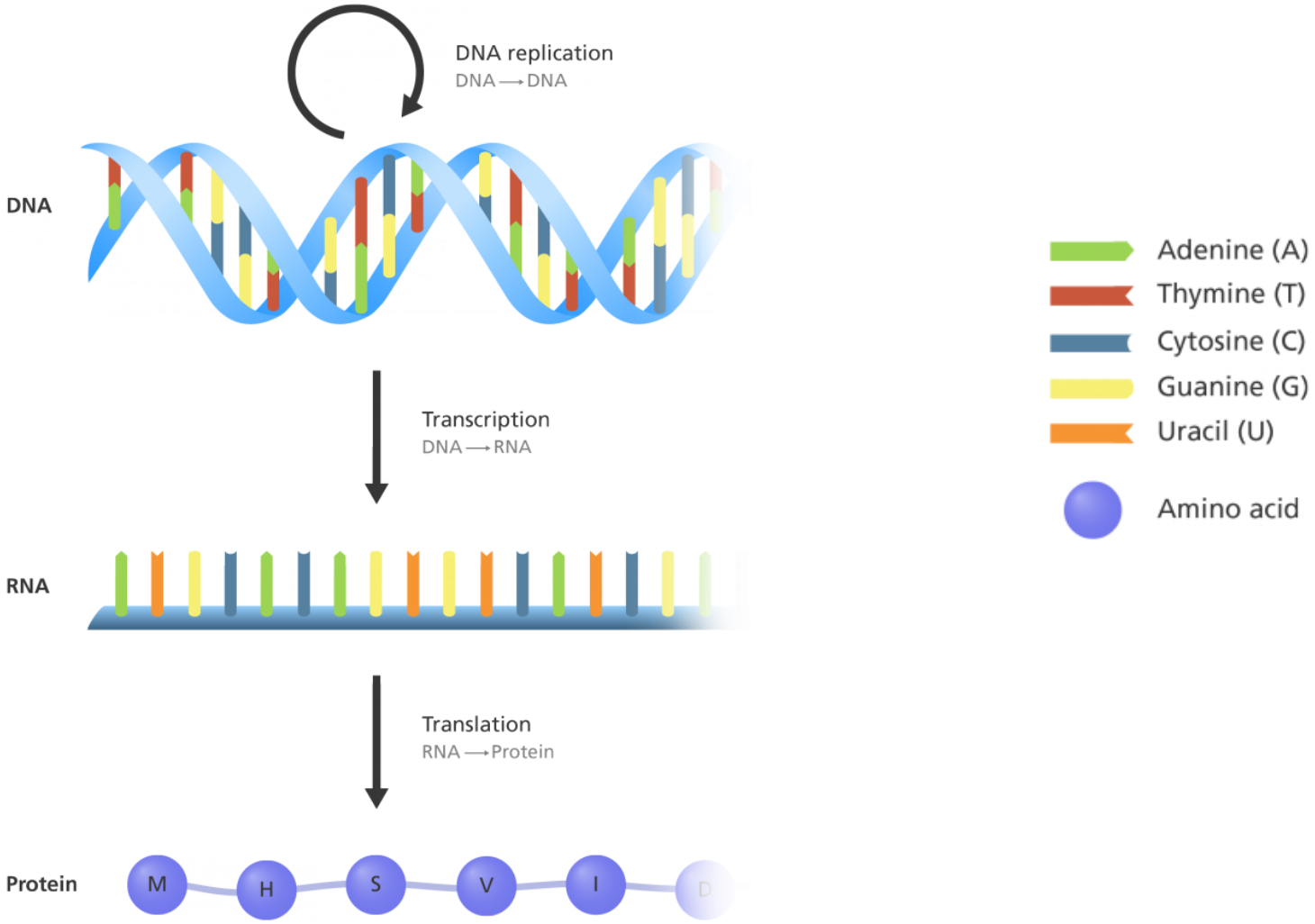
TP4: Reverse transcription

- Central Dogma of Molecular Biology
- Nobel prizes
- Reverse Transcriptase
- Performing a reverse transcription reaction
- Practical Applications

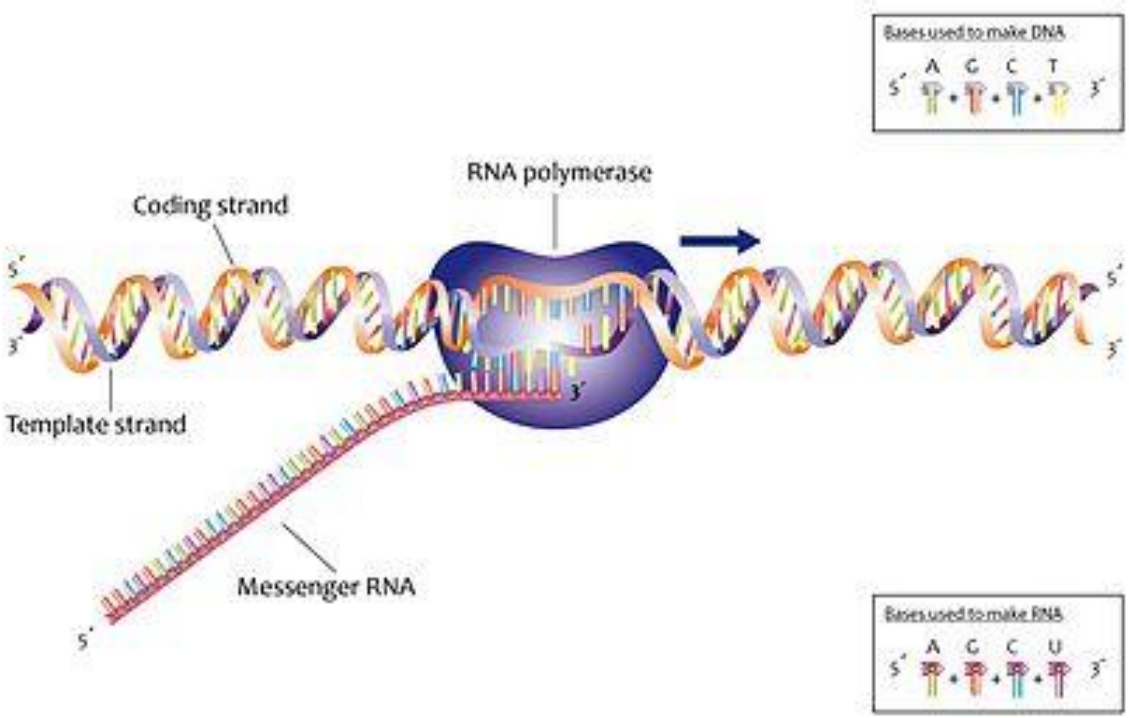
TP4: Reverse transcription

Central dogma of molecular biology



TP4: Reverse transcription

Central dogma of molecular biology



Transcription

DNA directed RNA synthesis

What is the biological significance?

Allows selective expression of genes

Regulation of transcription controls time, place and level of protein expression

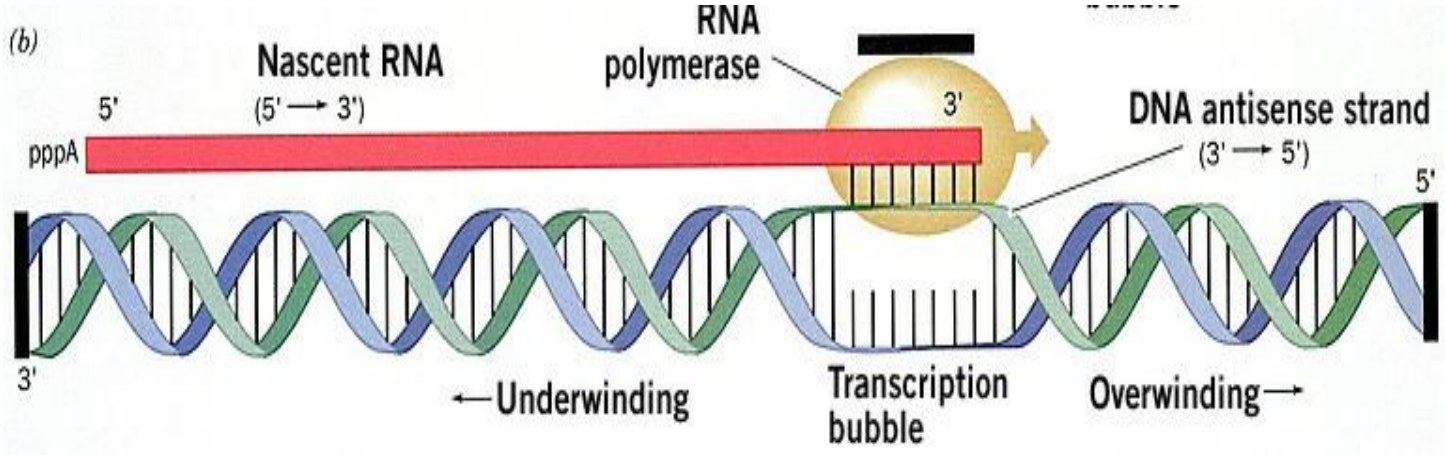
https://www.youtube.com/watch?v=5MfSYnItYvg

TP4: Reverse transcription

Central dogma of molecular biology

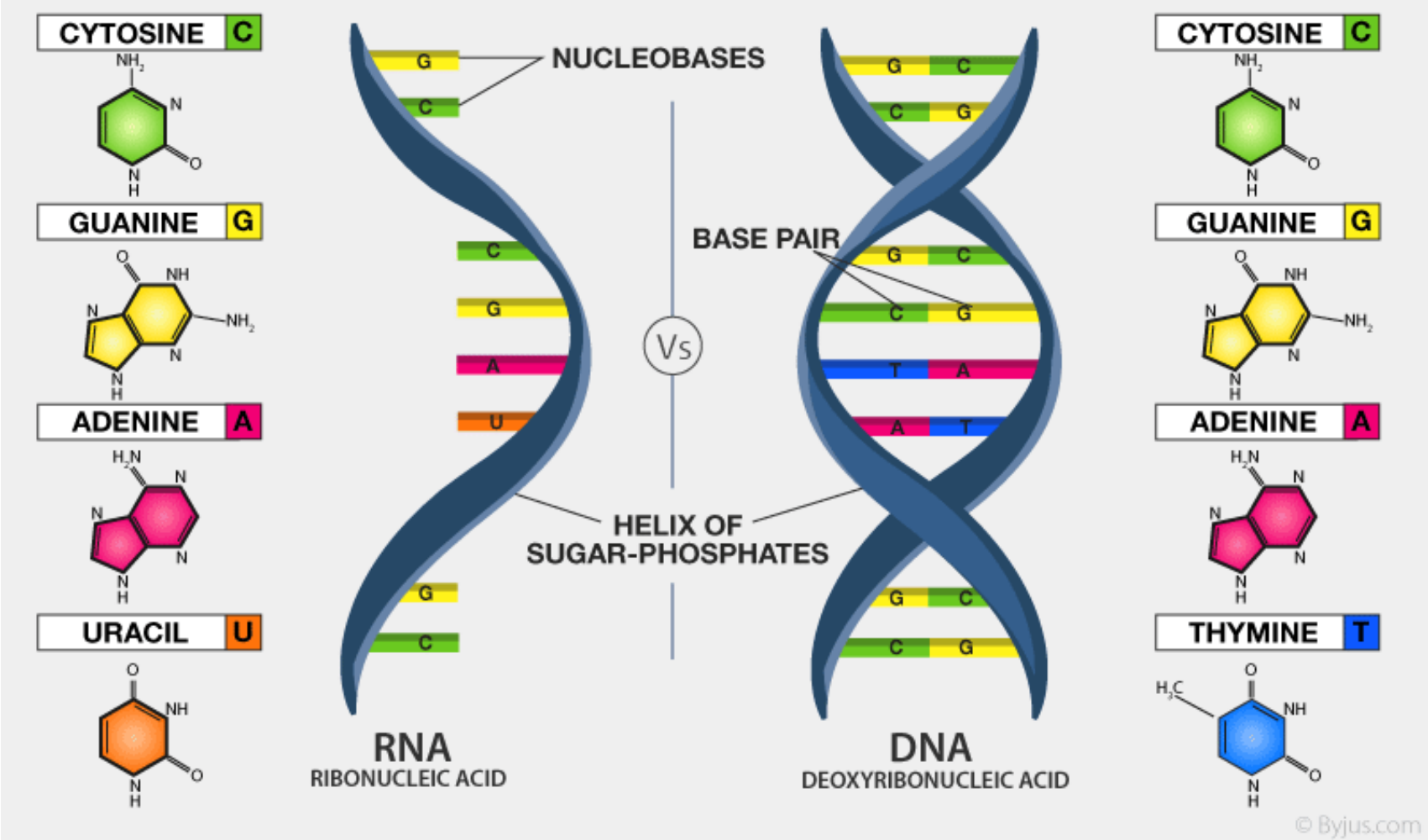
Transcription (DNA -> RNA)

Transcription is the mechanism by which a template strand of DNA is utilized by specific *RNA polymerases* to generate different types of RNA.



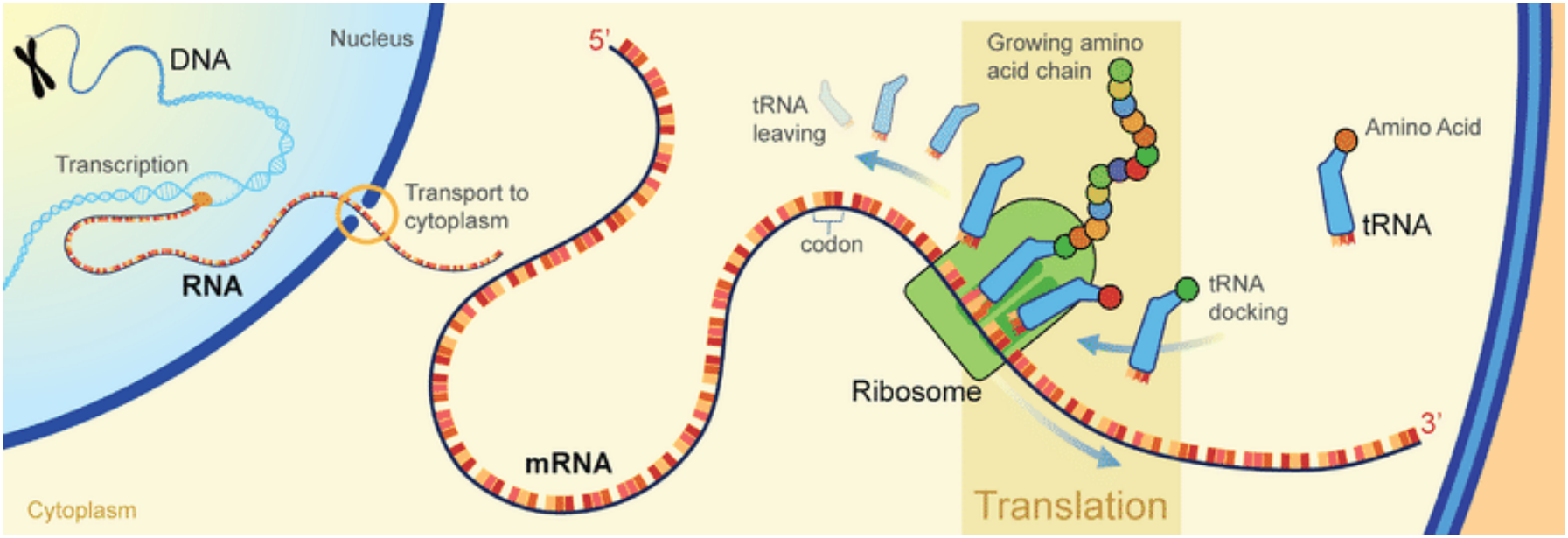
TP4: Reverse transcription

Central dogma of molecular biology



TP4: Reverse transcription

Central dogma of molecular biology



Translation → **RNA directed amino acid synthesis - proteins**

https://www.youtube.com/watch?v=gG7uCskUOrA

TP4: Reverse transcription

Retrovirus

•Any of a group of RNA viruses which insert a DNA copy of their genome into the host cell in order to replicate

•It was known that successful infection of cells by RNA tumor viruses required DNA synthesis.

•Scientists hypothesized that a virus would need to transcribe its RNA genome into DNA and then insert this DNA into the host cell genome. Once incorporated into the host genome, the virus would be transcribed as though it were another gene and could produce more RNA virus from its DNA. The "DNA provirus hypothesis" developed by Howard Martin Temin

Proof of the mechanism whereby this DNA template was generated from the RNA genome of the infecting virus remained elusive.

TP4: Reverse transcription

Howard Temin and David Baltimore experiments

They showed that the RNA tumor viruses Rauscher Mouse Leukaemia Virus and Rous Sarcoma Virus both contained an enzyme that converted the viral RNA genome into an DNA.

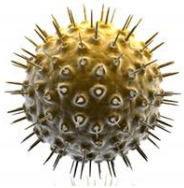
Can Isolated RNA Tumor Viral Particles Synthesize DNA?
To answer this question, viral particles were purified and incubated in a DNA synthesis reaction using a deoxynucleotide mix that included radiolabeled dTTP. An acid-insoluble product was generated, and the amount of product formed was proportional to the number of viral particles present.

Is the Reaction Product Really DNA?
To confirm the nature of the product of the polymerase reaction, both authors tested its sensitivity to DNase and RNase, confirming sensitivity to the former.

Is the Template RNA?
To confirm that the template is indeed the viral RNA, the polymerization reaction was performed in the presence and absence of ribonuclease (RNase). As expected, no DNA was synthesized when ribonucleases were included in the reaction.

Could the Observed Activity Be the Result of Conventional Polymerase Activity?
In both papers, the RNA tumor viruses R-MLV and Rous Sarcoma virus were also tested for the ability to incorporate rNTPs into acid-insoluble product. Neither was capable of synthesizing RNA, only showing polymerase activity when dNTPs were provided in the reaction mix.

TP4: Reverse transcription



•After Renato Dulbecco discovered that tumor viruses operate by incorporating their DNA into the DNA of host cells, Howard Temin and David Baltimore - independently of one another - discovered that viruses with genomes consisting of RNA can also be inserted into host cells' DNA.

•This takes place through an enzyme known as **"reverse transcriptase"**.

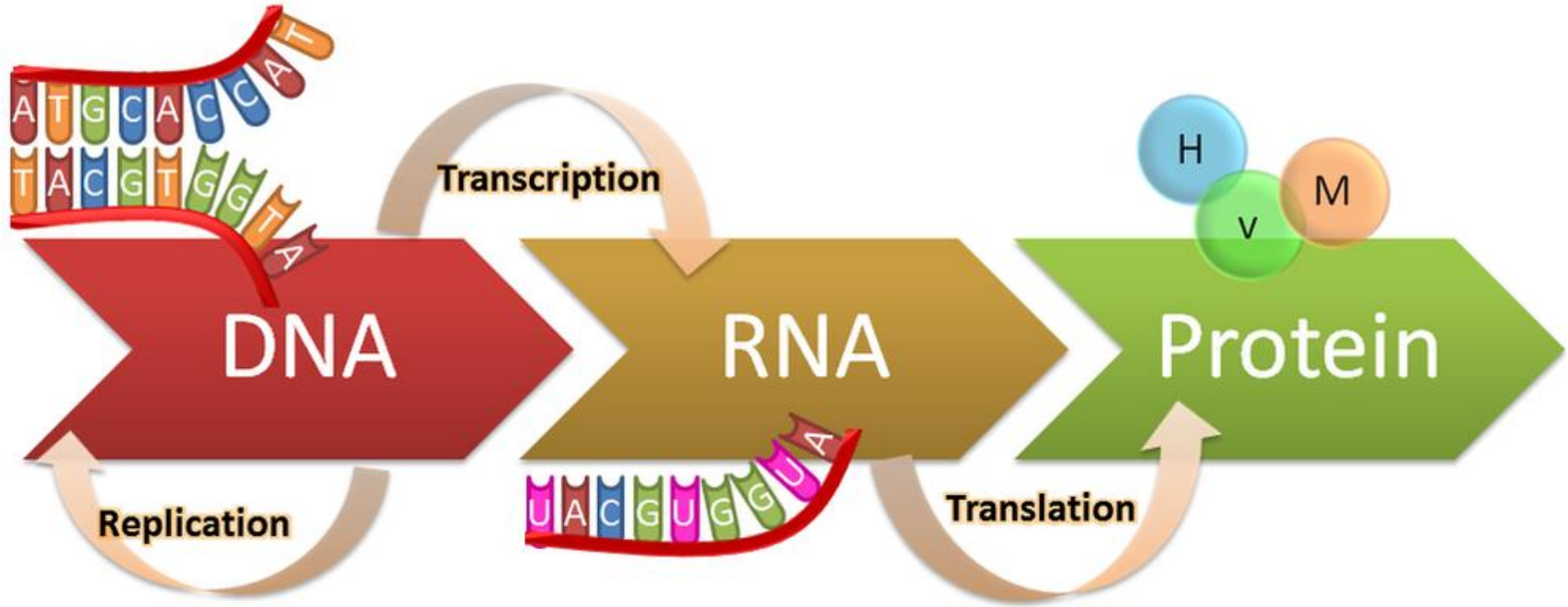
•Inside the host cell cytoplasm, the virus uses its own reverse transcriptase enzyme to produce DNA from its RNA genome. This new DNA is then incorporated into the host cell genome. The host cell then treats the viral DNA as part of its own genome, translating and transcribing the viral genes along with the cell's own genes, producing the proteins required to assemble new copies of the virus.

•The discovery that the information in RNA can be transferred to DNA meant that the generally accepted rule that genetic information was only transferred in one direction - from DNA to RNA, to protein - had to be modified.

The Central Dogma of Molecular Biology was challenged!

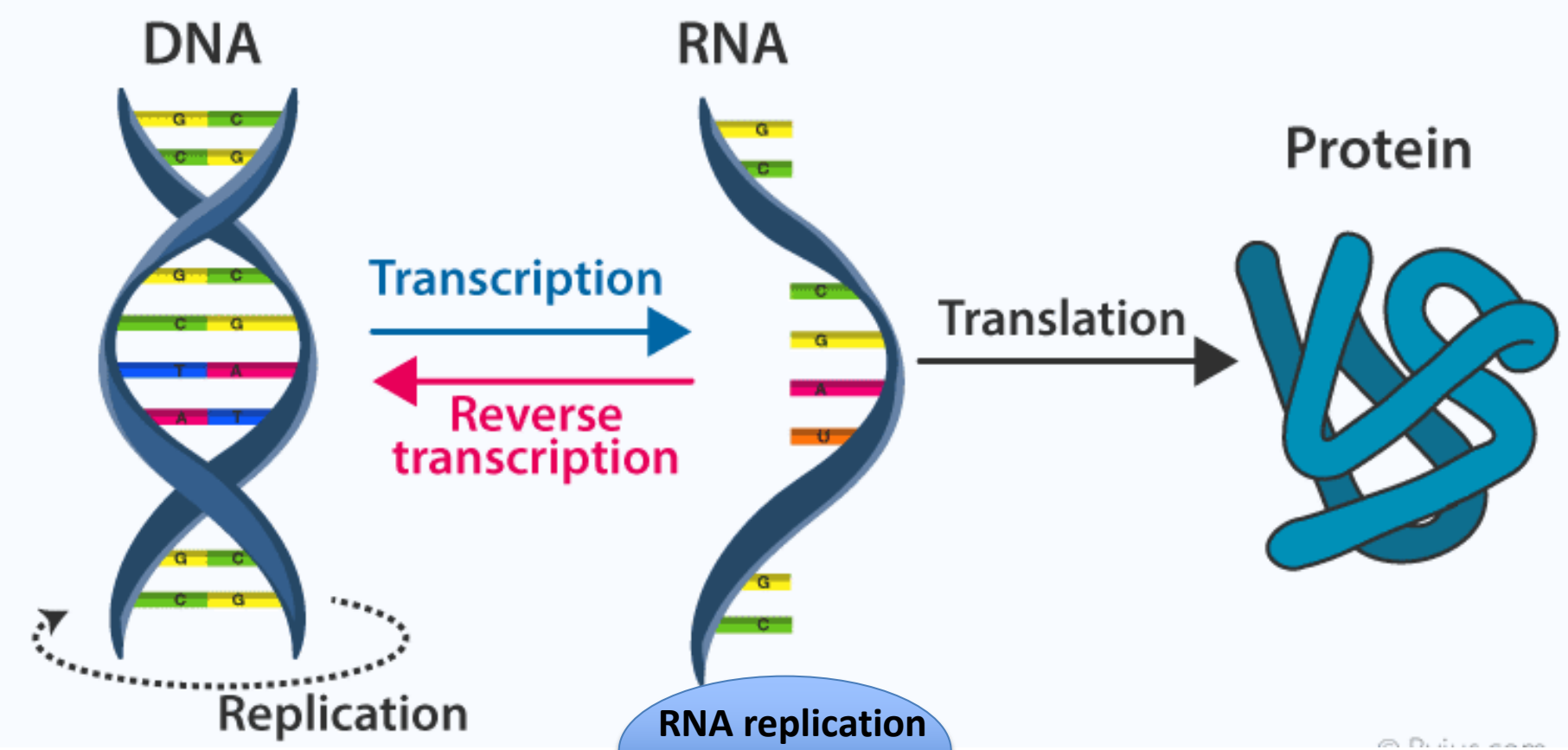
TP4: Reverse transcription

Central dogma of molecular biology was challenged



TP4: Reverse transcription

Central dogma of molecular biology was challenged



TP4: Reverse transcription

David Baltimore

Renato Dulbecco

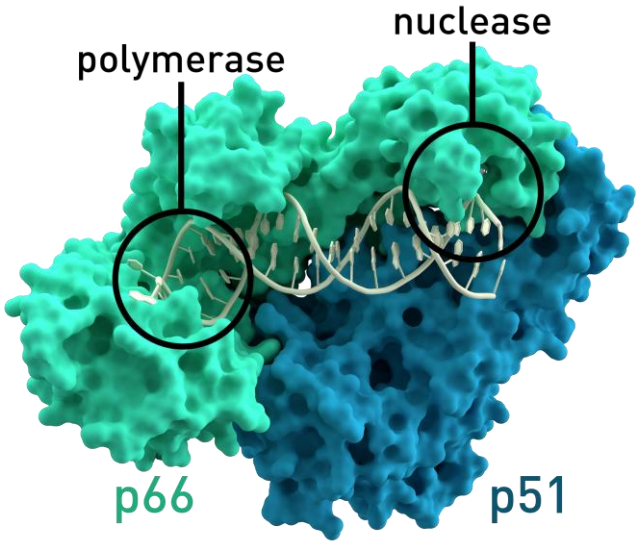
Howard Martin Temin

The Nobel Prize in Physiology or Medicine 1975 was awarded jointly to David Baltimore, Renato Dulbecco and Howard Martin Temin *"for their discoveries concerning the interaction between tumour viruses and the genetic material of the cell"*.

TP4: Reverse transcription

Reverse Transcriptase

Reverse transcriptases (RT) are enzymes composed of distinct domains that exhibit different biochemical activities. **RNA-dependent DNA polymerase activity** and **RNase H activity** are the predominant functions of reverse transcriptases. But they may also present DNA-dependent DNA polymerase activity.



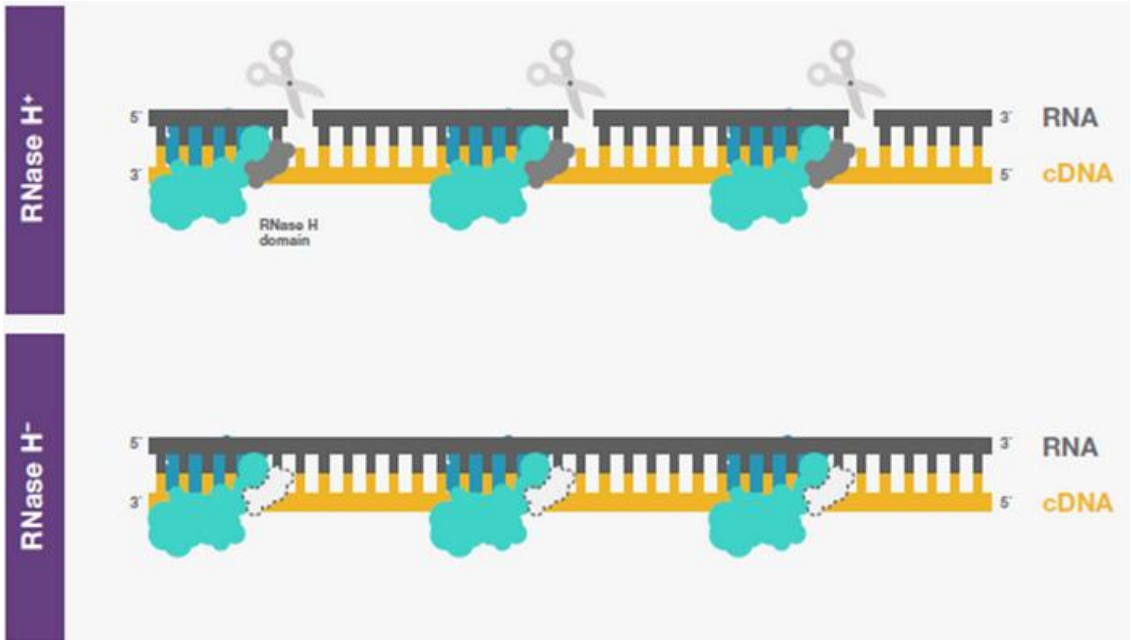
Retroviral RT has 3 sequential biochemical activities:

1. RNA-dependent DNA polymerase activity
2. Ribonuclease H (RNase H), degrades RNA from RNA-DNA duplexes to allow efficient synthesis of double-stranded DNA)
3. DNA-dependent DNA polymerase activity

TP4: Reverse transcription

Reverse Transcriptase RNase activity

RNase H activity cleaves the RNA template of the RNA: cDNA hybrid concurrently with polymerization.



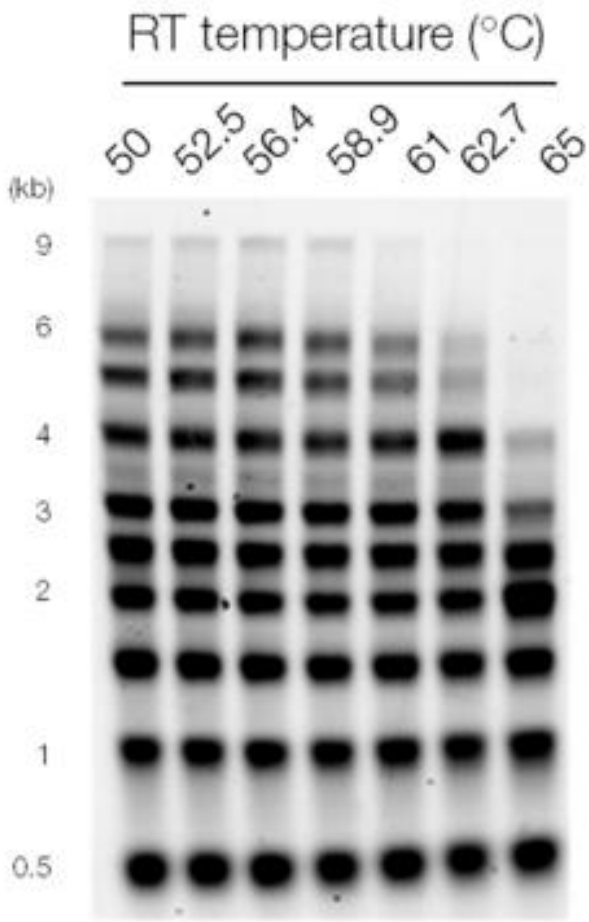
TP4: Reverse transcription

Reverse Transcriptase Thermostability

Elevated reaction temperatures help denature RNA with strong secondary structures and/or high GC content, allowing reverse transcriptases to read through the sequence. As a result, reverse transcription at higher temperatures enables full-length cDNA synthesis and higher yields, which leads to better representation of an RNA population by the cDNAs.

- Wild-type AMV reverse transcriptase – 42-48ºC
- Wild-type MMLV reverse transcriptase - 37°C.
- Engineered MMLV reverse transcriptases -up to 55°C

Such highly thermostable reverse transcriptases are especially suitable to synthesize cDNA from GC-rich RNA templates.



TP4: Reverse transcription

Reverse Transcriptase Fidelity

The fidelity of reverse transcriptase represents sequence accuracy maintained by the enzyme during synthesis of DNA from RNA. Fidelity is inversely correlated to an error rate of reverse transcription. MMLV-based reverse transcriptases are reported to have an error rate in the range of one in 15,000 to 27,000 nucleotides synthesized, with AMV reverse transcriptase displaying an even higher error rate

TP4: Reverse transcription

Reverse Transcription step-by-step

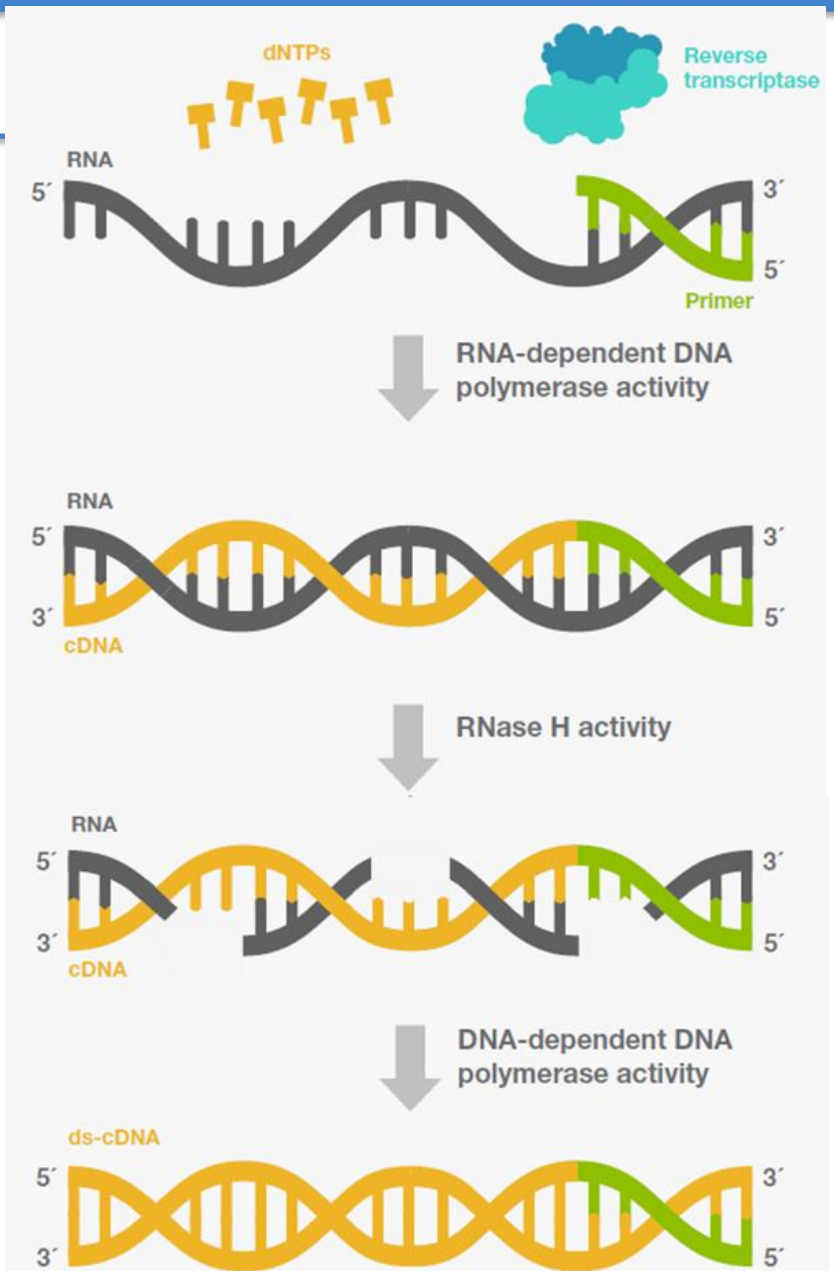
•In the presence of an annealed primer, reverse transcriptase binds to an RNA template and initiates the reaction.

•RNA-dependent DNA polymerase activity synthesizes the complementary DNA (cDNA) strand, incorporating dNTPs.

•RNase H activity degrades the RNA template of the DNA:RNA complex.

•DNA-dependent DNA polymerase activity (if present) recognizes the single-stranded cDNA as a template, uses an RNA fragment as a primer, and synthesizes the second-strand cDNA.

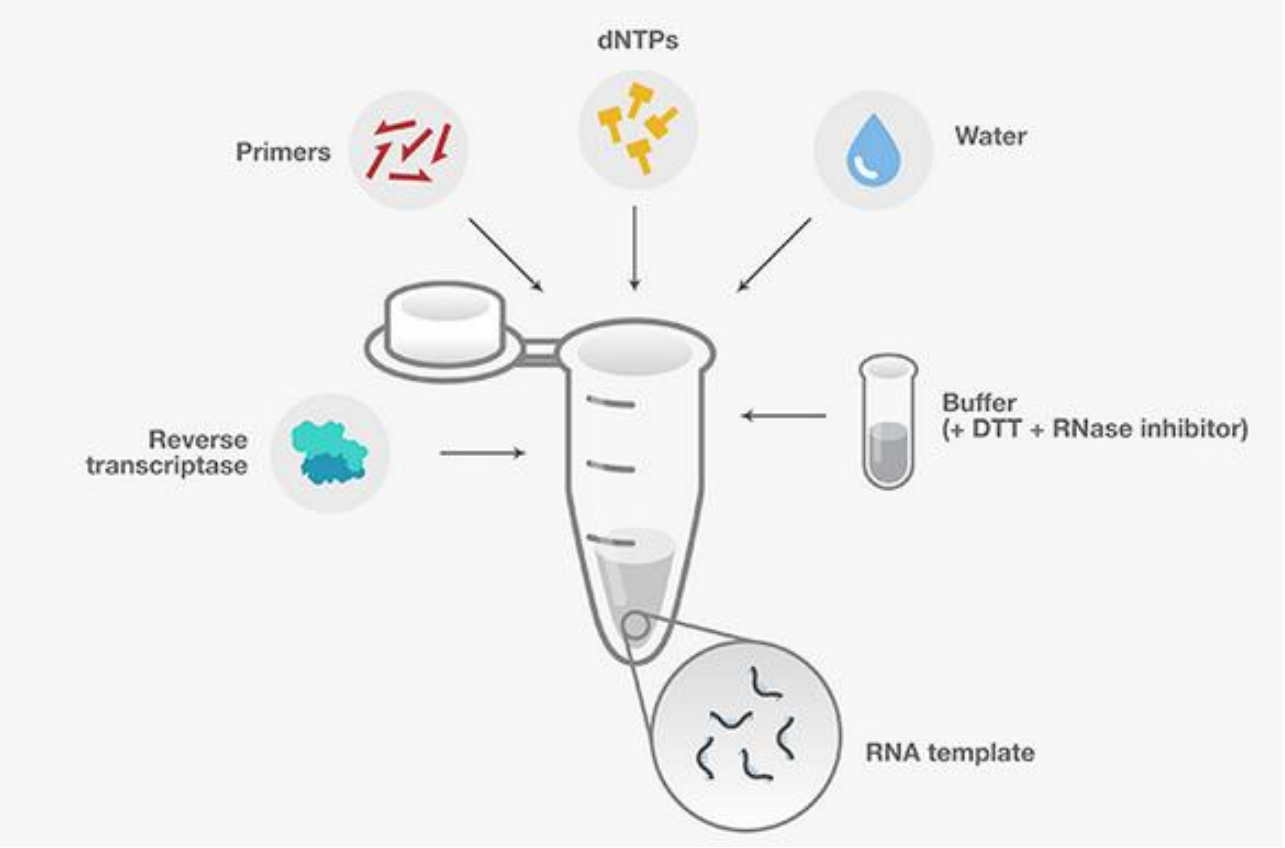
•Double-stranded cDNA is formed.



TP4: Reverse transcription

How to prepare a reverse transcription reaction?

- Water
- Buffer
- RNA template
- Primers
- Nucleotides
- Reverse transcriptase
- DNA Polymerase
- RNase inhibitor



TP4: Reverse transcription

Reverse Transcription step-by-step

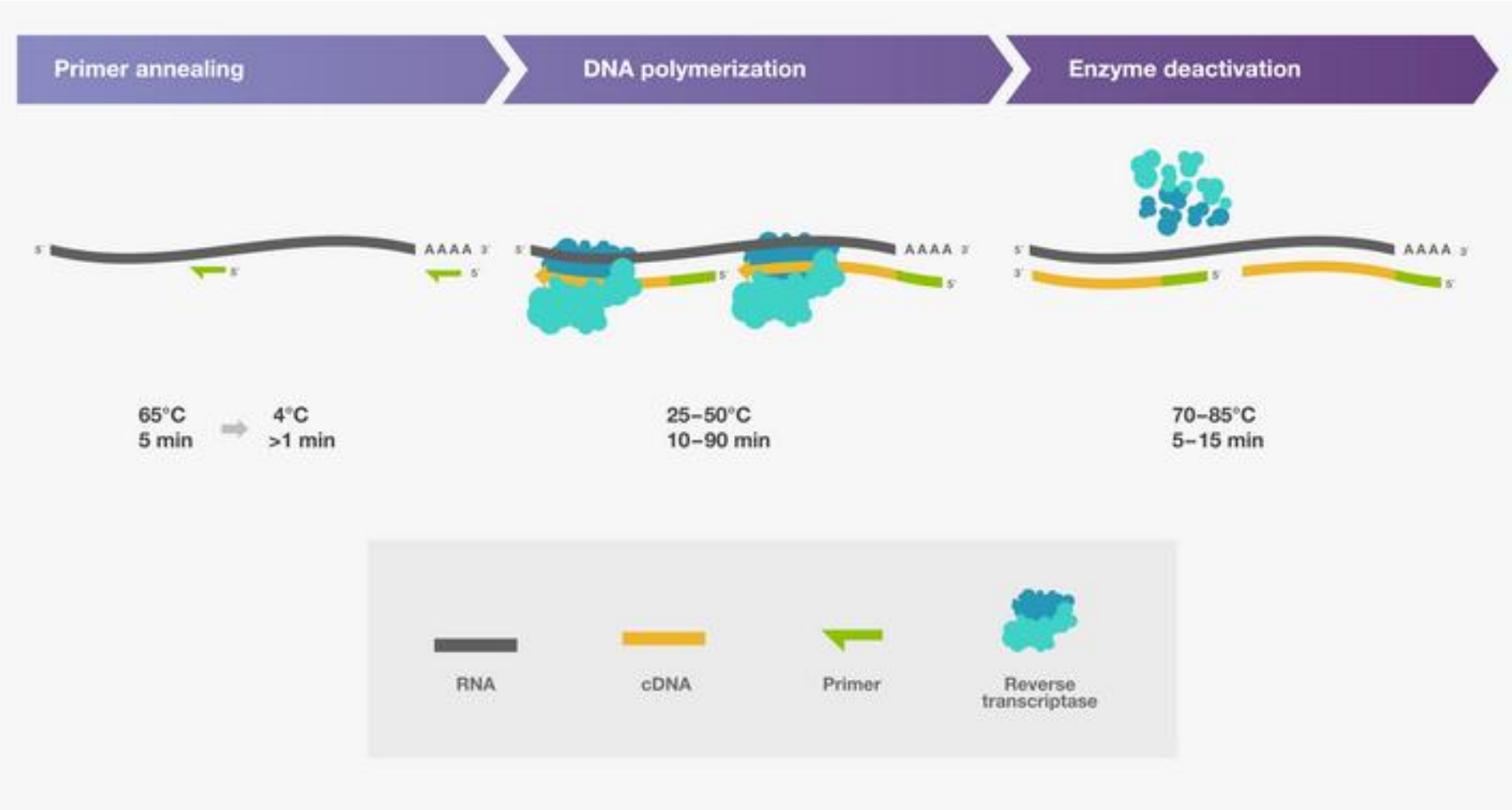
1- Primer annealing: The primer is mixed with the RNA template, heated to 65°C for 5 min, then incubated on ice for at least 1 min. This helps ensure that the RNA is single-stranded and that the primer anneals to the target efficiently.

2-After annealing, the reverse transcriptase and necessary components (e.g., buffer, dNTPs, RNase inhibitor) are added.

3-DNA polymerization: In this step, reaction temperature and duration may vary according to the primer choice and reverse transcriptase used. With an oligo (dT) primer (Tm ~35–50°C), the reaction may be incubated directly at the optimal temperature of the reverse transcriptase (37–50°C). Random hexamers typically have lower Tm (~10–15°C) due to their shorter length.

TP4: Reverse transcription

Reverse Transcription step-by-step



https://www.youtube.com/watch?v=0MJIbrS4fbQ

TP4: Reverse transcription

Reverse Transcription step-by-step

DNA (template) 3’- TACTAACTTAGCCTCGCATCA-5’

mRNA 5’- AUGAUUGAAUCGGAGCUAGU-3’

cDNA 3’- TACTAACTTAGCCTCGCATCA-5’

Coding DNA 5’- ATGATTGAATCGGAGCGTAGT-3’

TP4: Reverse transcription

Reverse Transcription step-by-step

DNA (template) 3’- TACTAACTTAGCCTCGCATCA-5’

mRNA **5’- AUGAUUGAAUCGGAGCUAGU-3’**

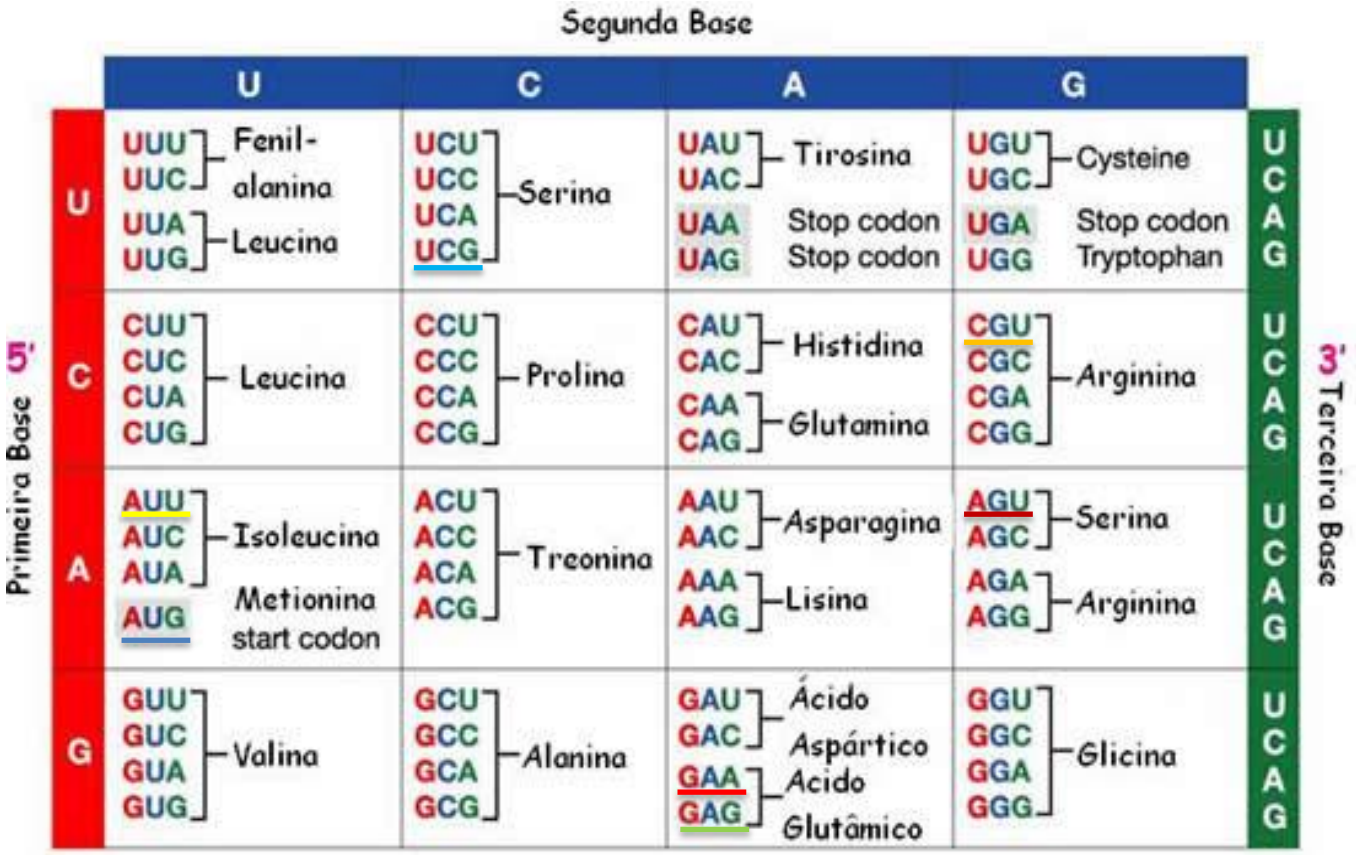
cDNA 3’- TACTAACTTAGCCTCGCATCA-5’

Coding DNA **5’- ATGATTGAATCGGAGCGTAGT-3’**

TP4: Reverse transcription

Reverse Transcription step-by-step

Coding DNA 5’- ATGATTGAATCGGAGCGTAGT-3’



TP4: Reverse transcription

Reverse Transcription step-by-step

DNA (template) 3’- TACTAACTTAGCCTCGCATCA-5’

mRNA 5’- AUGAUUGAAUCGGAGCUAGU-3’

cDNA 3’- TACTAACTTAGCCTCGCATCA-5’

Coding DNA 5’- ATGATTGAATCGGAGCGTAGT-3’

Polypeptide NH2’- Met-Ile-Glu-Ser-Glu-Arg-Ser- COOH’

Applications

TP4: Reverse transcription

-Use of Reverse Transcriptase Inhibitors as antiviral drugs as RT structure is different between eukaryotes, prokaryotes and viruses

-Production of useful molecules using recombinant DNA technology

-RT-PCR applications for:

- Gene expression studies
- Analysis of RNA sequences
- Diagnosis of infectious agents
- Diagnosis of genetic diseases

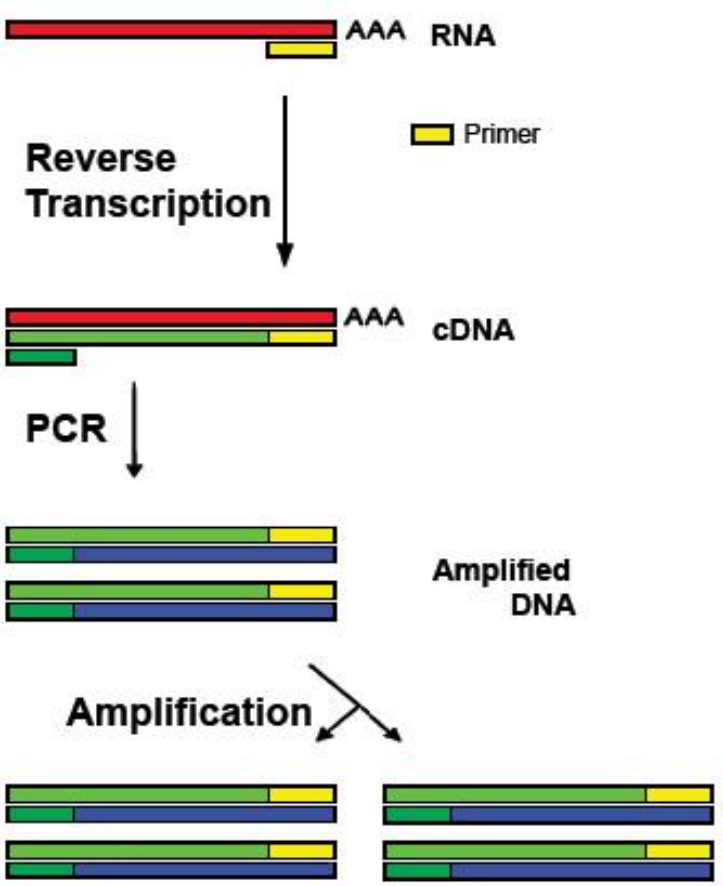
TP4: Reverse transcription

RT-PCR

RT-PCR is a variant of PCR used to detect RNA expression

While traditional PCR is used to exponentially amplify target DNA sequences, RT-PCR is used to clone expressed genes by reverse transcribing the RNA of interest into its DNA complement through the use of reverse transcriptase.

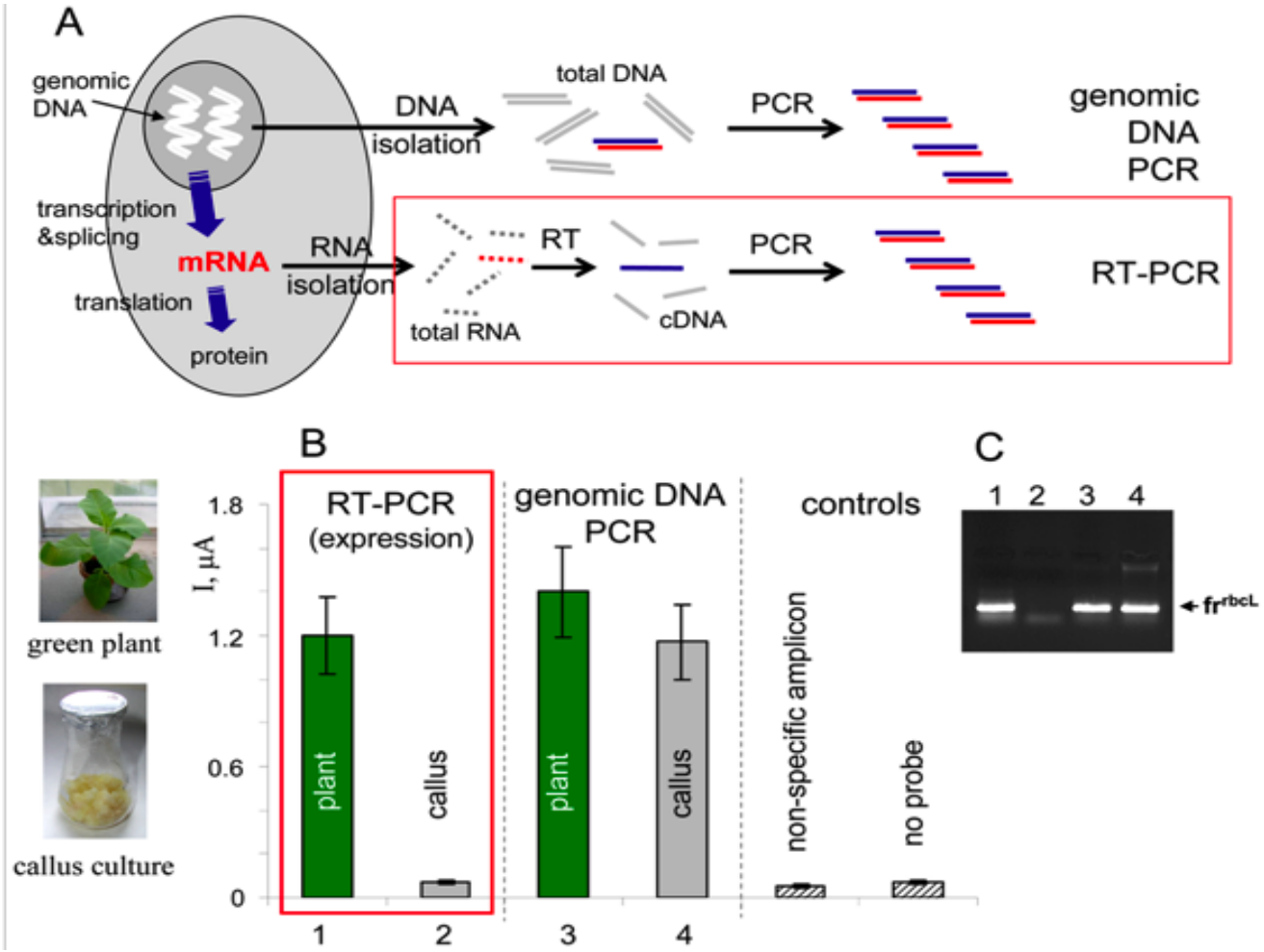
Subsequently, the newly synthesized cDNA is amplified using traditional PCR.



https://www.youtube.com/watch?v=JGoGhp6vcSE

TP4: Reverse transcription

RT-PCR



rbcL gene expression in *Nicotiana tobacum* cells (green plant vs plant callus)

rbcL-gene for RuBisCO large subunit

http://www.mdpi.com/1424-8220/8/1/193/htm

Laboratorial classes

Laboratorial

	4 e 18 Nov, 2 Dez	11 e 25 Nov, 9 Dez	4 e 18 Nov, 2 Dez	11 e 25 Nov, 9 Dez
2ªf 10.00-13.00	P6	P9	Mónica Sebastiana	Susana Serrazina
3ªf 9.00-12.00	P2	P1	Filipa Monteiro	Fernando Vaz Dias
4ªf 9.00-12.00	P12	P11	Mónica Sebastiana	Fernando Vaz Dias
4ªf 17.00-20.00	P10	P3	Ana Rita Santos	Susana Serrazina
5ªf 10.30-13.30	P8	P5	Filipa Monteiro	Fernando Vaz Dias
6ªf 14.00-17.00	P4	P7	Mónica Sebastiana	Susana Serrazina

Docentes:

Mónica Sebastiana: mgsebastiana@fc.ul.pt
Rita Santos: absantos@fc.ul.pt
Filipa Monteiro: fimonteiro@fc.ul.pt
Fernando Dias: fmdias@fc.ul.pt
Susana serrazina: smserrazina@fc.ul.pt

Laboratorial

Boas Práticas

Laboratorial

Comportamento em laboratório

Vou começar a trabalhar num laboratório…..
O que devo saber?

✓Erros humanos e mau comportamento podem comprometer os melhores laboratórios e medidas de proteção pessoais

Informa-te sobre os riscos do teu trabalho

Conhece as regras e as normas de funcionamento de cada laboratório– *manual de boas práticas laboratoriais*

Atenção à sinalização de segurança

✓Outros aspetos importantes: bata, caderno de laboratório, equipamentos, conduta, ética

Laboratorial

Usar óculos protetores sempre que necessário
Especial cuidado com o uso de lentes de contacto – podem absorver produtos químicos e provocar lesões

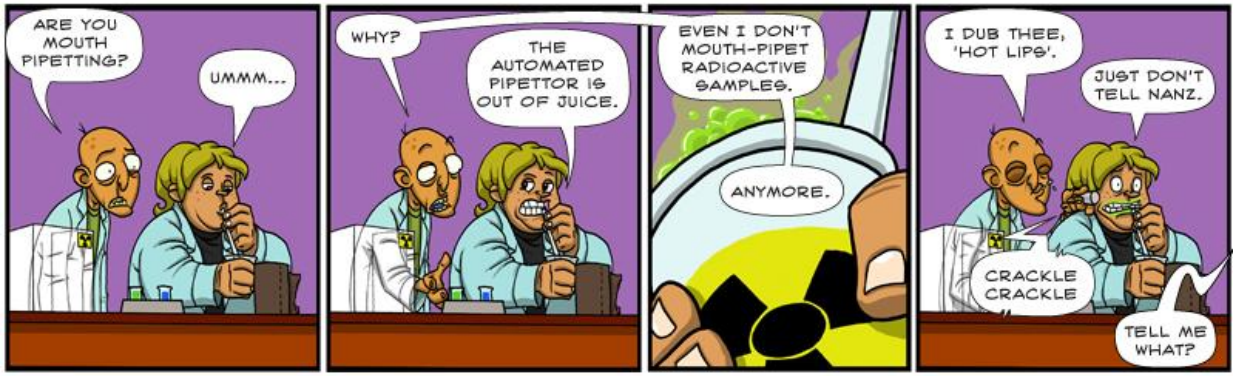
Caminhar com atenção, não correr no laboratório

Para utilizar os equipamentos, lê e compreende as instruções de manuseamento e segurança

Laboratorial

Conhece as propriedades físicas e toxicidade dos reagentes antes de iniciar a experiência.

Nunca pipetar com a boca.
Não levar a mão à boca se estiver a manusear reagentes.



Laboratorial

Não deixar equipamento de laboratório ou reagentes inflamáveis perto de uma chama

Segue corretamente o protocolo da experiência.
Improvisações podem provocar acidentes.
Para cada experiência devemos fazer um planeamento adequado.

Laboratorial

Cadernos de laboratório:

- *Qual foi a vossa última aula antes deste workshop?*
- *O que almoçaram ontem?*
- *No dia 13 de Março de 2018, onde estavam às 17h?*
- *O que jantaram no dia em que fizeram 15 anos?*

Como é nos lembramos de experiências passadas?

Registos – caderno de laboratório

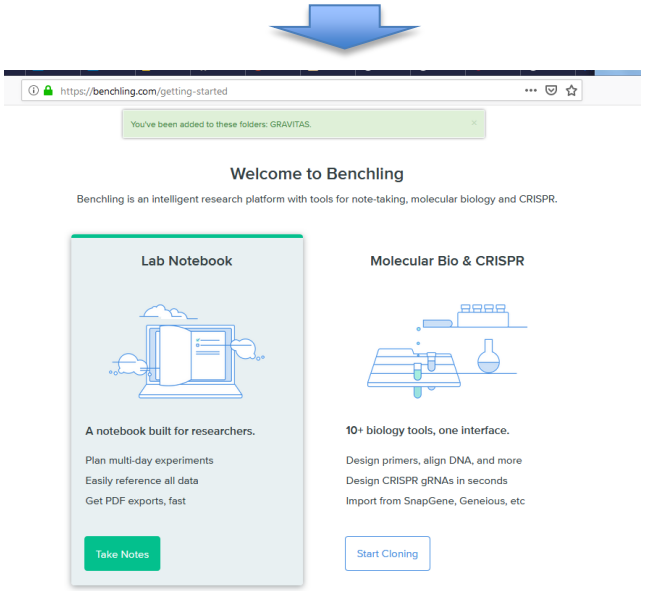
Laboratorial

Formato do caderno de laboratório:

- *Identificação: nome, ano, projeto. No interior deverá também constar o email, nome do supervisor, contactos*
- *As páginas dever, estar numeradas e com data!*

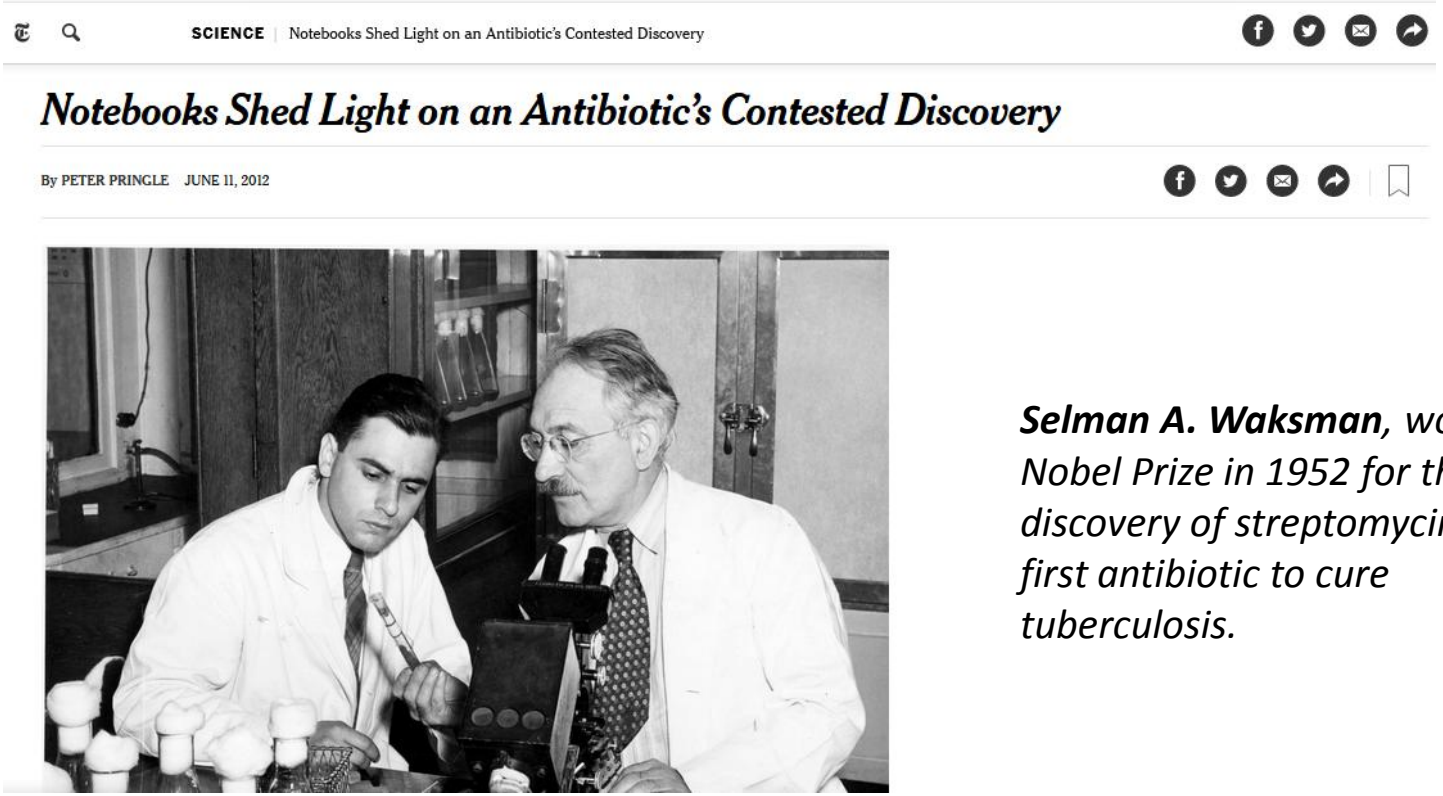
Rotina:

- *Fazer um registo diário*
- *Semanalmente, verificar se toda a informação importante foi registada*
- *Juntar fotos, gráficos*
- *Anotar informação a discutir com o supervisor*
- *Aderir às novas tecnologias – GO DIGITAL!*



Laboratorial

Porquê manter um caderno de laboratório?



SCIENCE | Notebooks Shed Light on an Antibiotic's Contested Discovery

Notebooks Shed Light on an Antibiotic's Contested Discovery

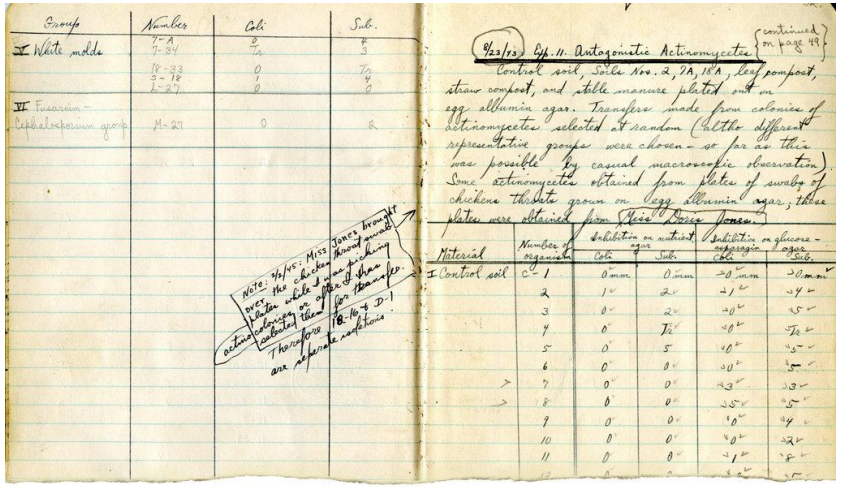
By PETER PRINGLE JUNE 11, 2012

***Selman A. Waksman**, won a Nobel Prize in 1952 for the discovery of streptomycin, the first antibiotic to cure tuberculosis.*

Laboratorial

Porquê manter um caderno de laboratório?

"When she pulled down the box and carefully opened it, however, there, loosely piled inside, were five clothbound notebooks — just like Dr. Waksman's, but marked "Albert Schatz."

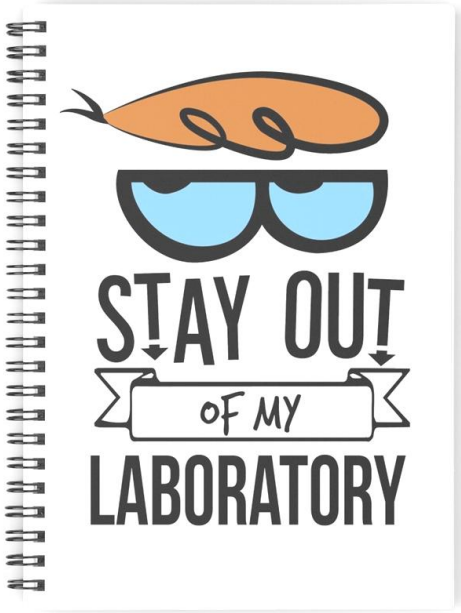


"In the notebook for 1943, on Page 32, Dr. Schatz had started Experiment 11. In meticulous cursive, he had written the date, Aug. 23, and the title, "Exp. 11 Antagonistic Actinomycetes," a reference to the strange threadlike microbes found in the soil that produce antibiotics. Underneath the title he recorded where he had found the microbes in "leaf compost, straw compost and stable manure" on the Rutgers College farm, outside his laboratory.
The following pages detailed his experiments and his discovery of two strains of a gray-green actinomycete named Streptomyces griseus, Latin for gray. Each strain produced an antibiotic that destroyed germs of E. coli in a petri dish — and, he was to find out later, also destroyed the TB germ. ***The notebook shows that the moment of discovery belongs to Dr. Schatz****."*

Laboratorial

De quem é o caderno de laboratório?

- **Não é teu!**
- O caderno de laboratório pertence ao laboratório e instituição/empresa,
- Deve ser retido pelo menos por 6-7 anos,
- O teu trabalho pode ser continuado,
- Poderás e deverás fazer a tua cópia!



Laboratorial

Conduta em laboratório – integridade científica

- **Fabricar:**

  "The intentional act of creating data or results that do not exist and for which there is no basis in fact, and recording and/or reporting them in the research record with the intention to mislead or deceive."

- **Falsificar:**

  "The practice of omitting or altering research data, materials, equipment or processes in such a way that the results of the research are no longer accurately reflected in the research record."

- **Plagiar:**

  "The intentional use of someone else's words, data, research as your own"

Laboratorial

Conduta em laboratório – planeamento

- Torna os teus objetivos num plano de ação – definição de tarefas
- Seleciona tarefas semanais: "*To do list*"
- Criar um calendário de planificação semanal para as atividades essenciais: laboratório, meetings, seminários, eventos sociais, etc
- Escolhe tarefas da tua lista e calendariza-as na tua planificação semanal
- Diariamente avalia se atingiste os teus objetivos e cria a tua planificação para o dia seguinte

Muito Importante!
O objetivo é obteres controlo e otimizares o teu tempo de forma a não te tornares escravo do trabalho!

Laboratorial

Micropipetas :

Escolher pipeta segundo o volume

P10
P200 (50/100)
P1000

Escolher pontas:
Branca
Amarela
Azul

Pipetar:
1: Pressionar embolo (1º stop)
2: Aspirar
3. Verter (2ª stop)

Usar caixas sem fita ou fita azul !