Reverse Transcriptase PCR

کاربرد ها: تشخیص آلودگی ویروس و آنالیز بیان ژن

مراحل انجام:

۱- استخراج Total RNA/mRNA از طریق متد AGT و یا کیت های مختلف استخراج RNA موجود در بازار (بررسی کمیت و کیفیت RNA استخراجی)

۲- تبدیل mRNA/total RNA به cDNA توسط آنزیم RT (RNA-dependent DNA ploymerase، RNAaseH و DNA-dependent DNA polymerase) (بررسی کیفیت cDNA)

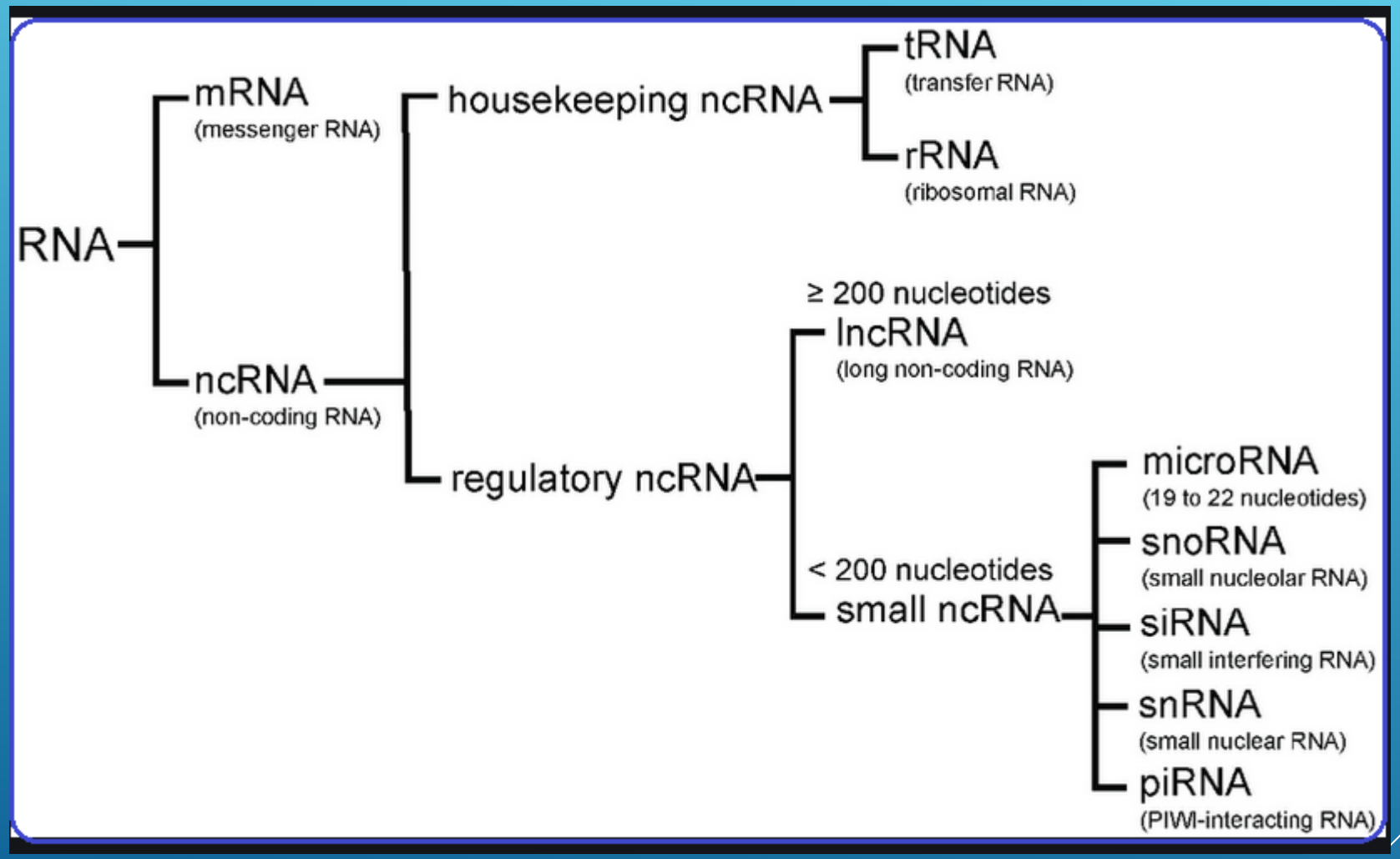
۳- انجام واکنش PCR در حضور cDNATemplate و نوکلئوتیدها و Taq pol و غیره

RT-PCR: one-step, two-step

مسائل مهم در RT-PCR

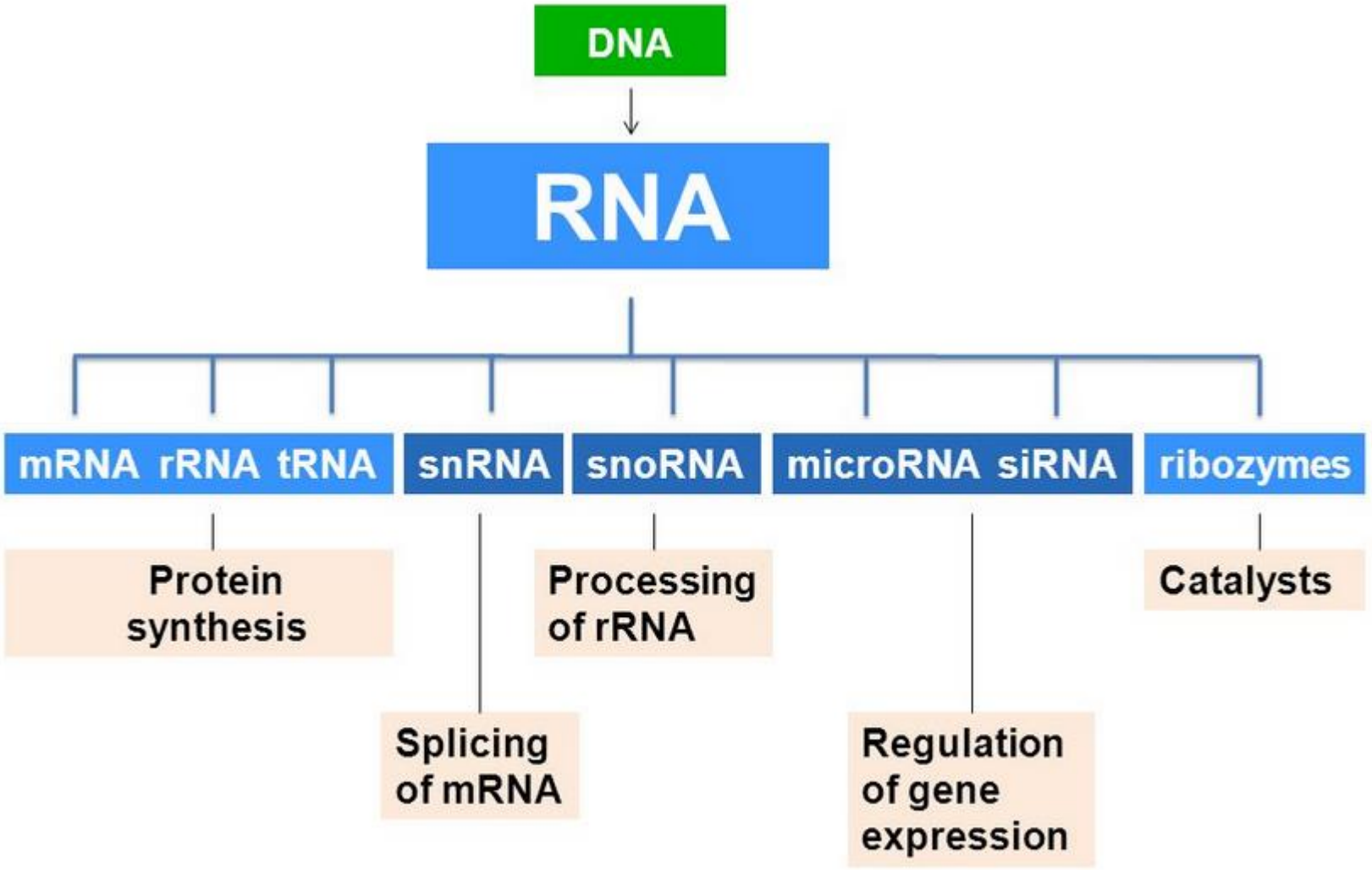
۱- آلودگی DNA (دو کنترل منفی)

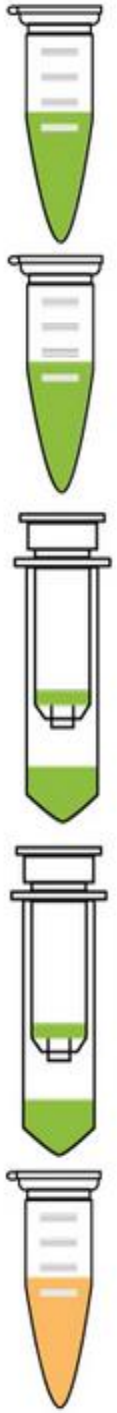
۲- کنترل های مثبت (Housekeeping genes)


RNA
mRNA
(messenger RNA)
ncRNA
(non-coding RNA)
housekeeping ncRNA
tRNA
(transfer RNA)
rRNA
(ribosomal RNA)
regulatory ncRNA
≥ 200 nucleotides
lncRNA
(long non-coding RNA)
< 200 nucleotides
small ncRNA
microRNA
(19 to 22 nucleotides)
snoRNA
(small nucleolar RNA)
siRNA
(small interfering RNA)
snRNA
(small nuclear RNA)
piRNA
(PIWI-interacting RNA)

RNA types & functions

Types of RNAs	Primary Function(s)
mRNA - messenger	translation (protein synthesis) regulatory
rRNA - ribosomal	translation (protein synthesis) <catalytic>
t-RNA - transfer	translation (protein synthesis)
hnRNA - heterogeneous nuclear	precursors & intermediates of mature mRNAs & other RNAs
scRNA - small cytoplasmic	signal recognition particle (SRP) tRNA processing <catalytic>
snRNA - small nuclear snoRNA - small nucleolar	mRNA processing, poly A addition <catalytic> rRNA processing/maturation/methylation
regulatory RNAs (siRNA, miRNA, etc.)	regulation of transcription and translation,


DNA
RNA
mRNA rRNA tRNA
snRNA
snoRNA
microRNA siRNA
ribozymes
Protein synthesis
Splicing of mRNA
Processing of rRNA
Regulation of gene expression
Catalysts



Cell harvesting from blood, mammalian, bacterial, or fungal cells

Cell Lysis

RNA binds to the columns

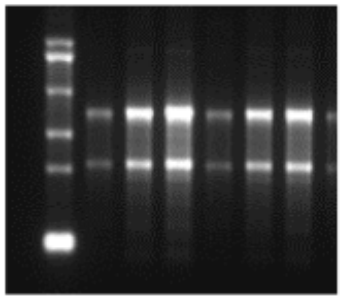
Wash

Elute RNA

Characterisation of the transcriptome

RNA sub-classes in a mammalian cell:

ribosomal RNA	rRNA	80-85%	*(5S, 18S und 28S)*
transfer RNA	tRNA	10-15%	
messenger RNA	**mRNA**	**1-5%**	
average length	1930 bases		
high abundant	<10 genes	10-20000 copies/cell	>1%
intermediate abundant	~500 genes	200-400 copies/cell	0,1%
low abundant	>10000 genes	<20 copies/cell	0.004%

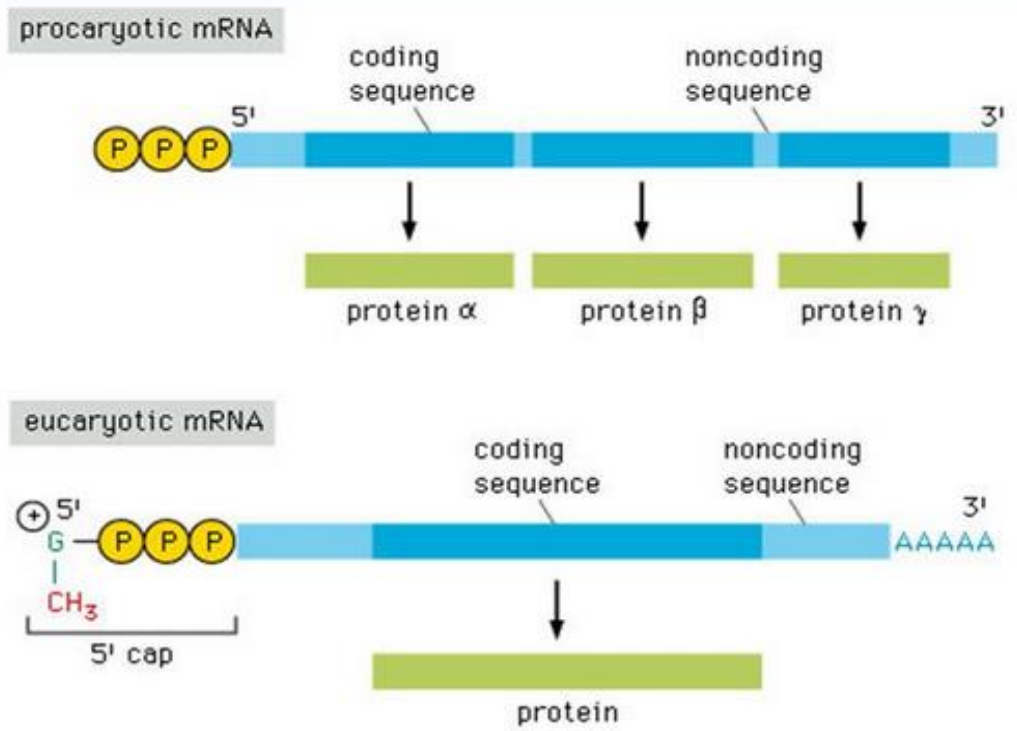


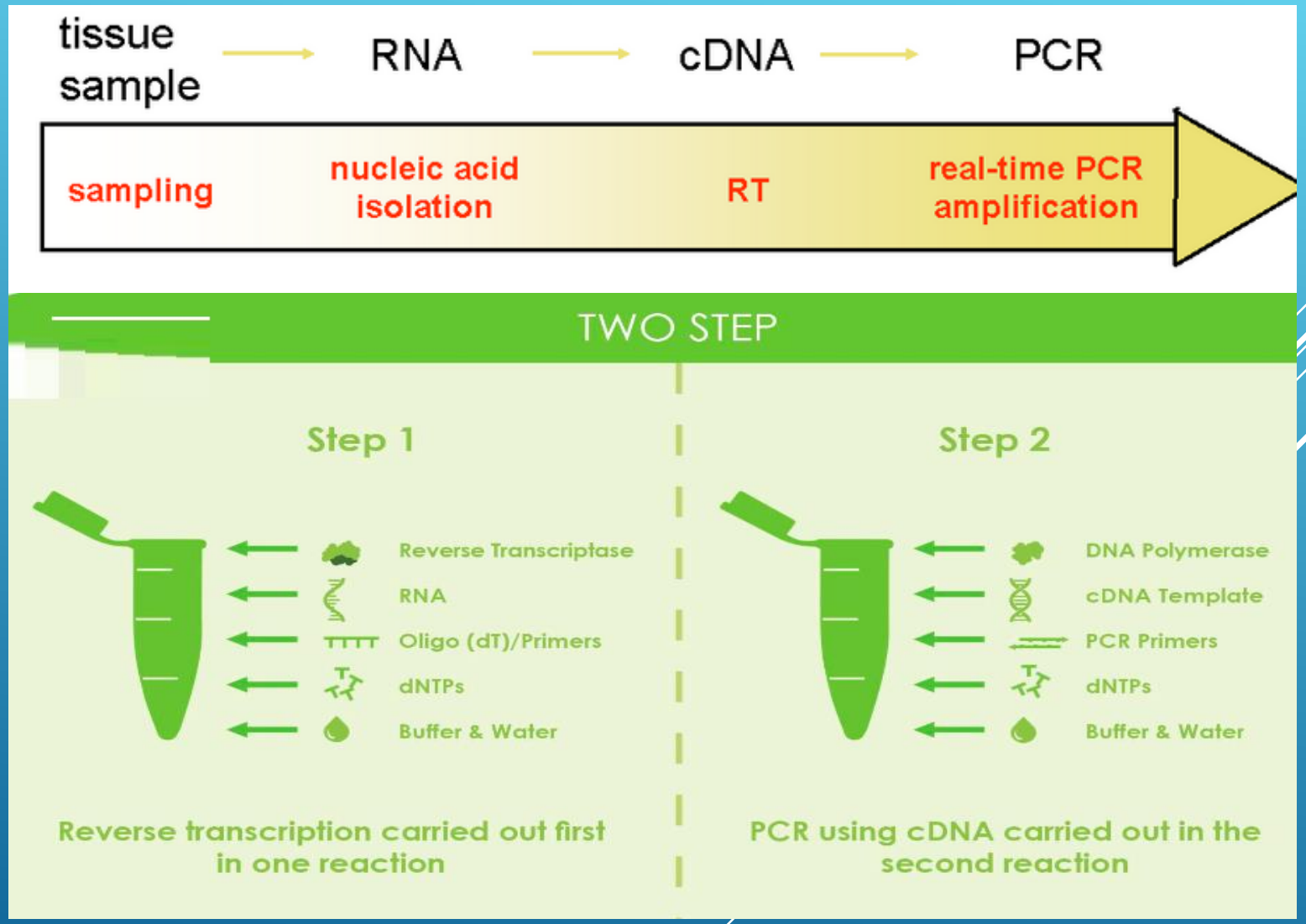
28S rRNA 3898-6333 bases

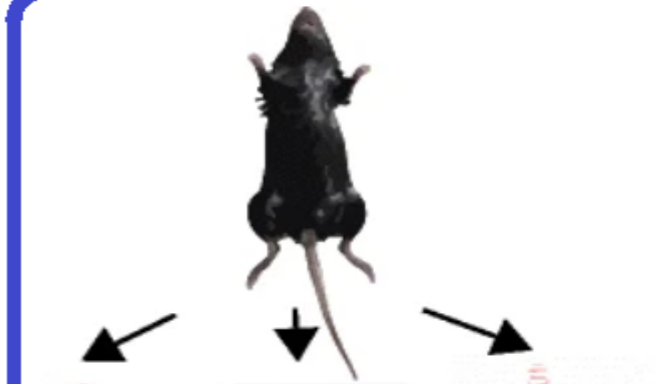
18S rRNA 1898–1976 bases

5S rRNA ~120 bases

Prokaryotic vs eukaryotic mRNA molecules




tissue sample
RNA
cDNA
PCR
sampling
nucleic acid isolation
RT
real-time PCR amplification
TWO STEP
Step 1
Reverse Transcriptase
RNA
Oligo (dT)/Primers
dNTPs
Buffer & Water
Reverse transcription carried out first in one reaction
Step 2
DNA Polymerase
cDNA Template
PCR Primers
dNTPs
Buffer & Water
PCR using cDNA carried out in the second reaction



Tissues homogenised in lysis buffers

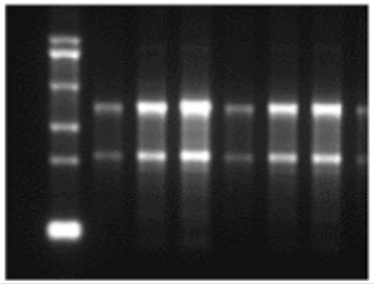
RNA extraction

- Isolate II
- miRVana
- Norgen total
- miRNeasy
- TRIzol

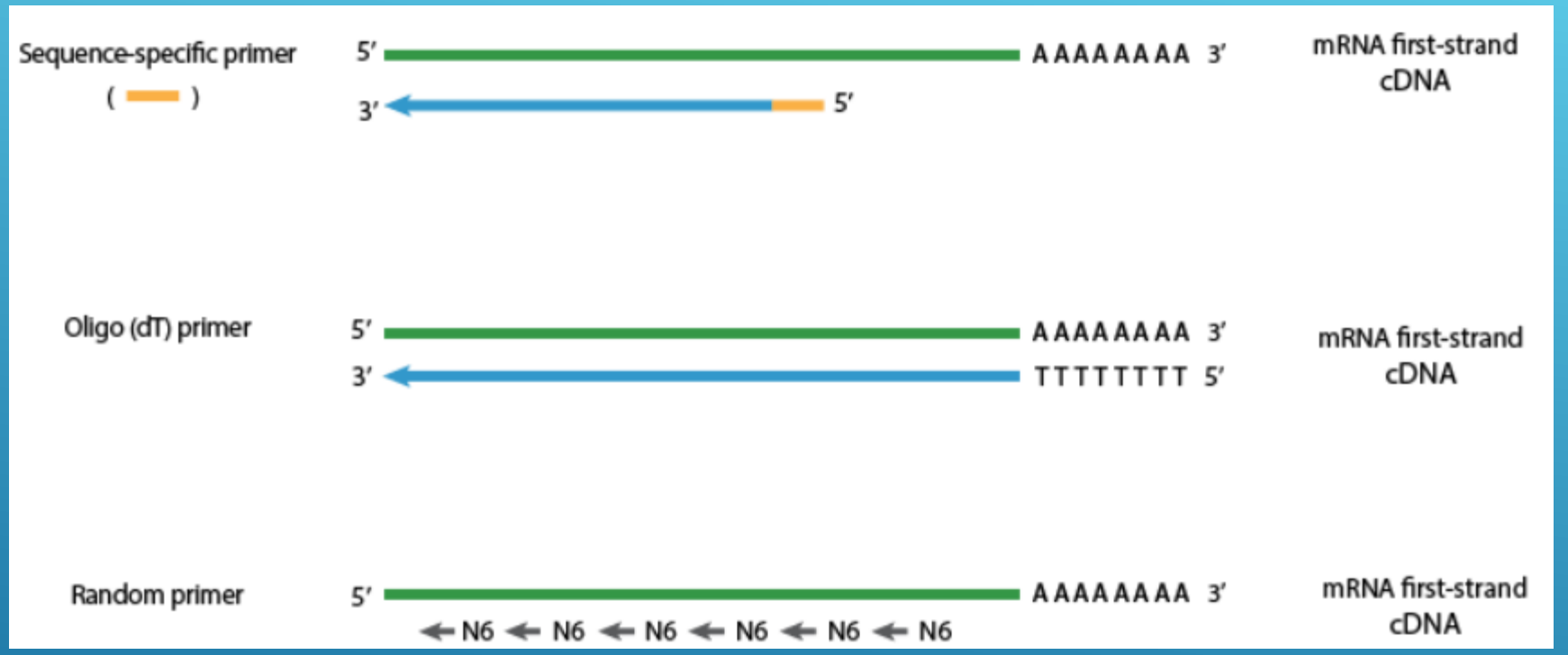
Characterisation of RNA by NanoDrop, Qubit and Agilent Bioanalyzer

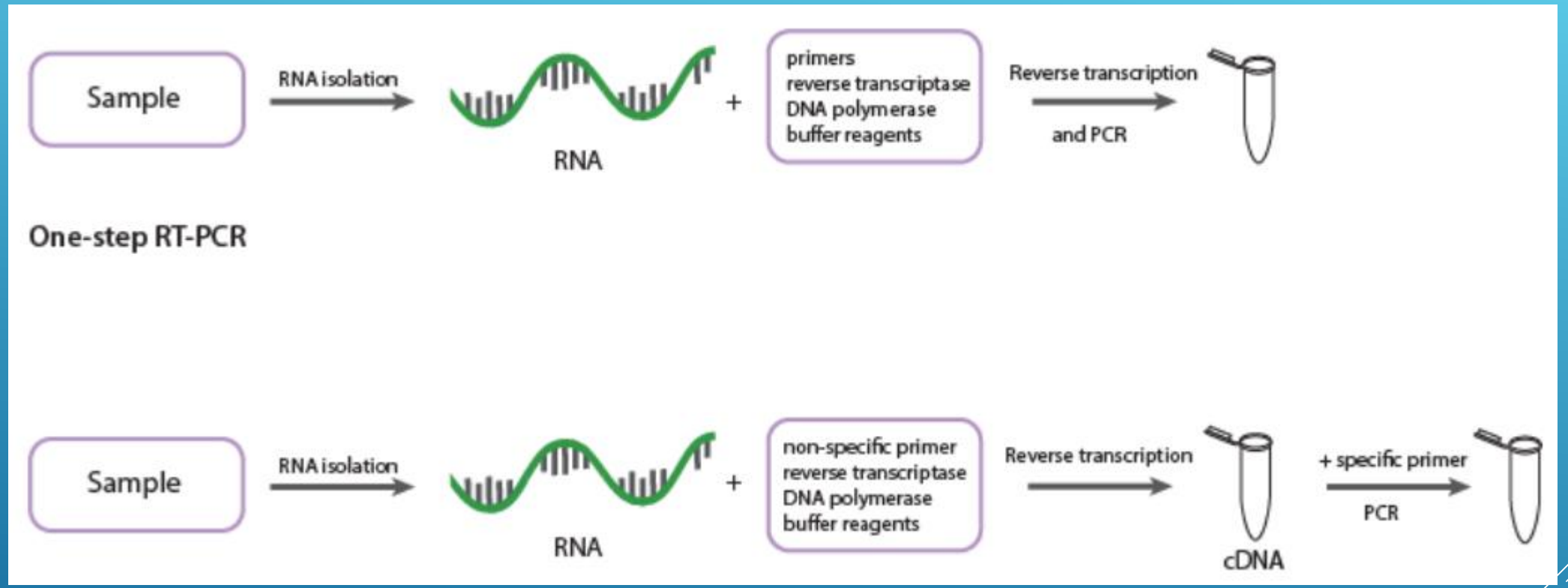
Total RNA was isolated using the following methods:

kits/ Acid guanidinium thiocyanate-phenol-chloroform (AGPC)



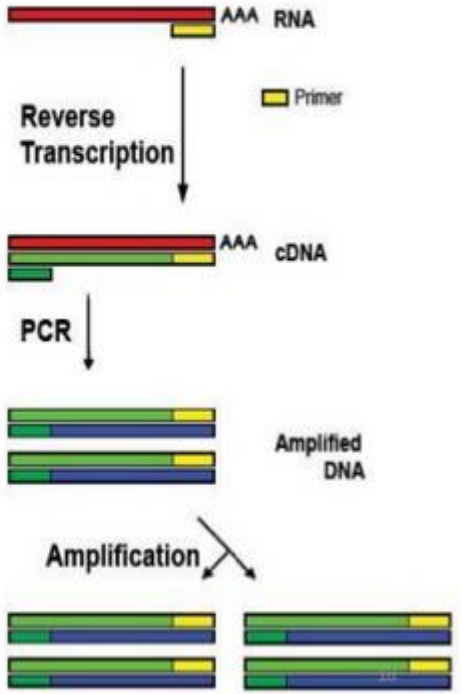
- **28S rRNA**
- **18S rRNA**
- **5S rRNA**

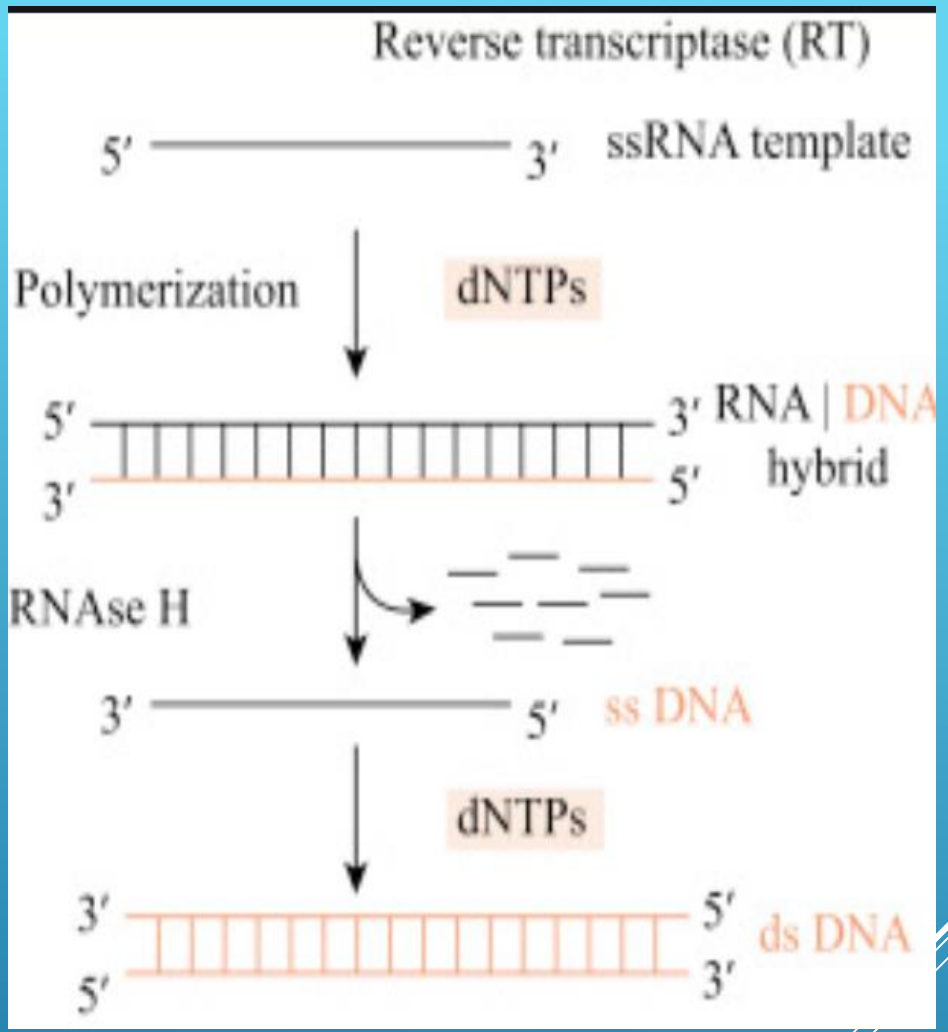

Sequence-specific primer
( )
5′
AAAAAAAA 3′
3′
5′
mRNA first-strand cDNA
Oligo (dT) primer
5′
AAAAAAAA 3′
3′
TTTTTTTT 5′
mRNA first-strand cDNA
Random primer
5′
AAAAAAAA 3′
N6 N6 N6 N6 N6 N6
mRNA first-strand cDNA


Sample
RNA isolation
RNA
+
primers
reverse transcriptase
DNA polymerase
buffer reagents
Reverse transcription
and PCR
One-step RT-PCR
Sample
RNA isolation
RNA
+
non-specific primer
reverse transcriptase
DNA polymerase
buffer reagents
Reverse transcription
cDNA
+ specific primer
PCR

REVERSE TRANSCRIPTION PCR (RT-PCR)

- for amplifying DNA from RNA.
- Reverse transcriptase reverse transcribes RNA into cDNA, which is then amplified by PCR.
- Some thermostable DNA polymerases used in the PCR such as Tth have a reverse transcriptase activity under certain buffer conditions.





Reverse Transcription:

- RNA transcript
- Choice of primers: oligo(dT), gene-specific or random
- 100 mM each of dNTPS (dATP, dTTP, dCTP, dGTP)
- Reverse transcriptase
- Reverse transcription buffer (details included below)
- DEPC treated or nuclease free water
- RNase inhibitor (optional – RNAse H can interfere with synthesis (10))

PCR:

- DNA Template
- Taq DNA polymerase
- DEPC treated or nuclease free water
- 100 mM each of dNTPS (dATP, dTTP, dCTP, dGTP)
- Forward and reverse primers

Materials for performing gel electrophoresis after PCR is completed:

- Agarose
- TE buffer
- Loading dye
- Ethidium Bromide

RT Buffer composition:

- 50 mM Tris-HCl (pH 8.3)
- 250 mM KCl
- 5 mM MgCl2
- 10 mM DTT (depending on exact protocol, this may not be included)br

Sample Protocol:

Two-step RT-PCR

1. Isolate RNA and design desired primers
2. RNA must be denatured – heat 2 µg of RNA at 65˚C for 5 minutes
3. Put denatured RNA on ice and setup tube for reverse transcription
    a. 2 µg RNA
    b. 20 µl RT buffer
    c. 2.5 µl dNTP mix
    d. 2.5 µM Primer (random, oligo(dT), or gene-specific)
    e. 2.5 U Reverse transcriptase
    f. Remaining amount to make a total 50 µl reaction - DEPC treated or nuclease free wate
4. Put in a thermocycler for 1 hour at 37-42˚C (the temperature may vary depending on the RTase used)
5. Denature the single stranded DNA by incubating the tube at 95˚C for 2 minutes. Place on ice

6. Setup PCR reaction

a. 2.5-10 µl RT reaction product

b. 5 µl 10X PCR buffer

c. 1 µl Forward primer

c. 1 µl Reverse primer

e. 2.5 µl dNTP mix

f. 0.5 µl Taq DNA polymerase

g. Top reaction up to 50 µl with PCR water

7. Run in thermocycler as follows:

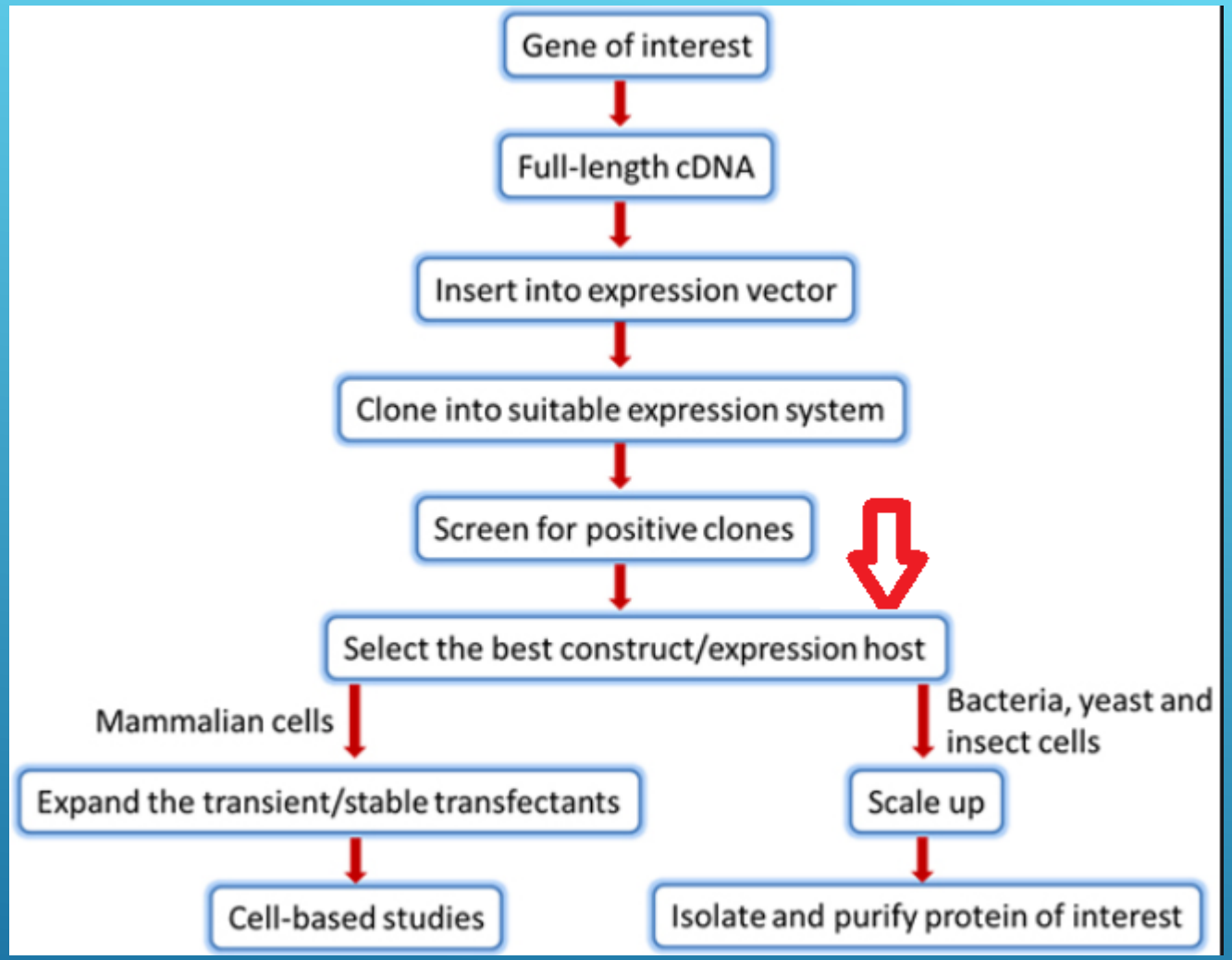
a. Denautration 98˚C - 30 seconds

b. 25-30 cycles:

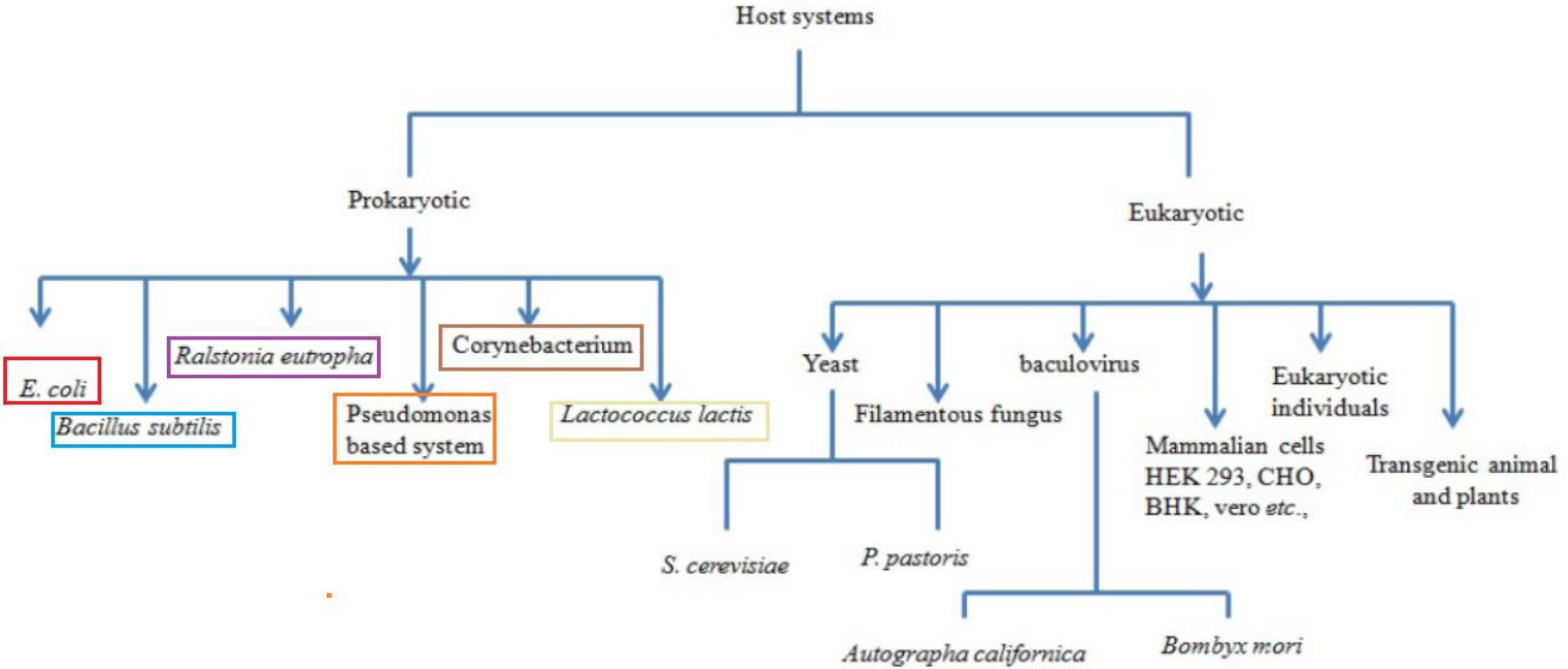
Step	Temperature	Time
Denature	98˚C	10 Seconds
Anneal	50-65 ˚C (depends on the Tm of primers)	30 Seconds
Extension	72˚C	Depends on primer length 1 minute per Kb

c. Final steps

Final Extension	72˚C	10 minutes
Hold	4˚C	Hold


Gene of interest
Full-length cDNA
Insert into expression vector
Clone into suitable expression system
Screen for positive clones
Select the best construct/expression host
Mammalian cells
Bacteria, yeast and insect cells
Expand the transient/stable transfectants
Scale up
Cell-based studies
Isolate and purify protein of interest

Heterologous and Homologous Expression of Proteins



Different host systems available for the production of recombinant Proteins

علت تنوع میزبان های پروکاریوتی:

۱- non-pathogenic و non-toxigenic

۲- sec-system

۳- growth on a wide variety of substrates

۴- competence cell

Table I: Comparison of cell-based protein expression systems

Expression System	Ease of Handling and Scale-Up*	Protein Expression Level	Cytotoxic Mammalian Proteins	Percent Yield (Based on Dry Weight)	PTMs†	Applications
Bacterial	****	Up to 10–30 g/L	Yes	1–5%	+	Functional assays Structural analysis Antibody generation Protein interactions
Yeast	***	Up to 30 g/L	Yes	1%	++	Functional assays Structural analysis Antibody generation Protein interactions
Insect	**	Up to 500 mg/L	Yes	30%	+++	Functional assays Structural analysis Antibody generation
Mammalian	*	Under 10 mg/L		<1%	++++	Functional assays Protein interactions Antibody generation

*Most difficult handling: ****; easiest handling: *.
†Very minimal PTM: +; PTM the closest to that in naturally occurred proteins: ++++.

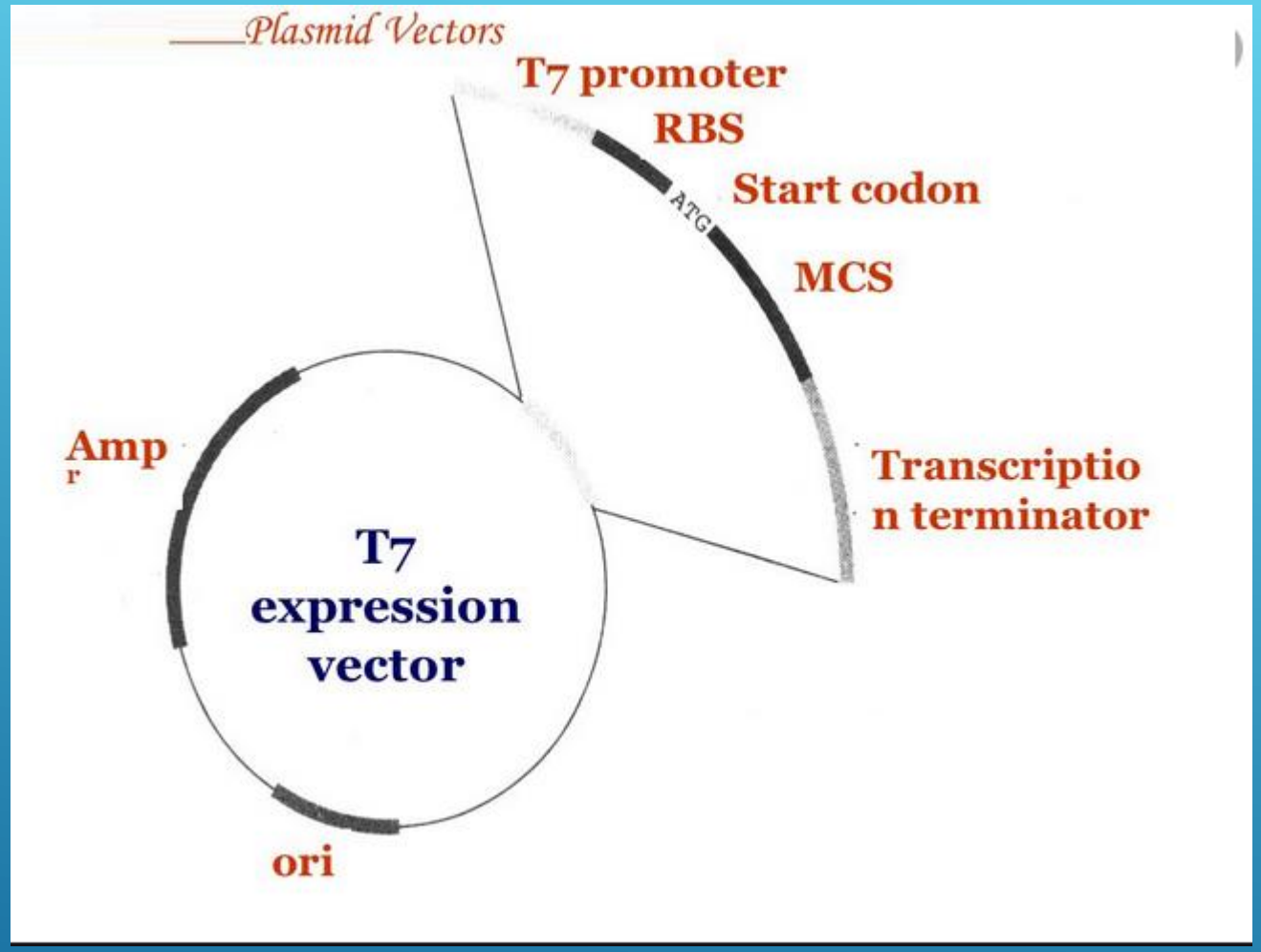
معایب اصلی میزبان های پروکاریوتی بعنوان سیستم بیان پروتئین های هترولوگوس

۱- PTM=post-translational modifications

۲- Folding (نیازمند آنزیم دی سولفید ایزومراز و سیستم چاپرونی GRP78/calnexin/calreticulin)

۳- Splicing

۴- Cutting

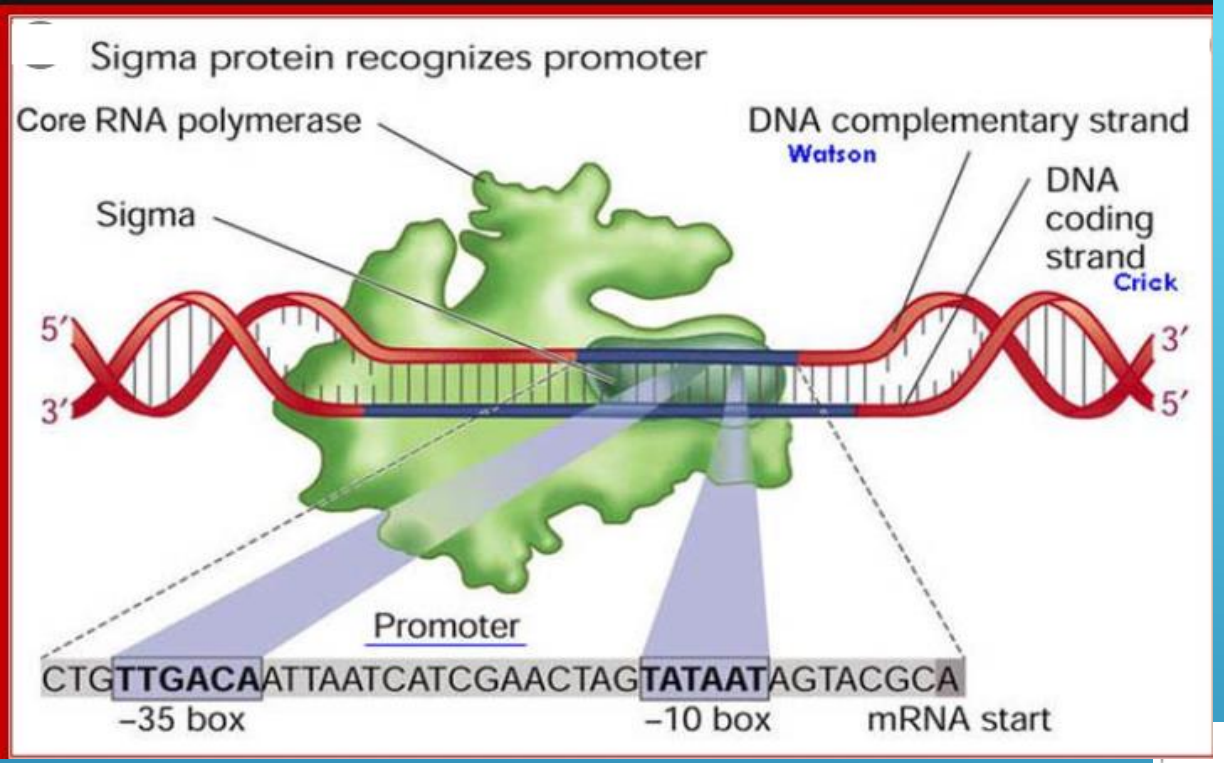


بیان ژن های بیگانه در E. coli

۱- Promoter

۲- Terminator

۳- RBS



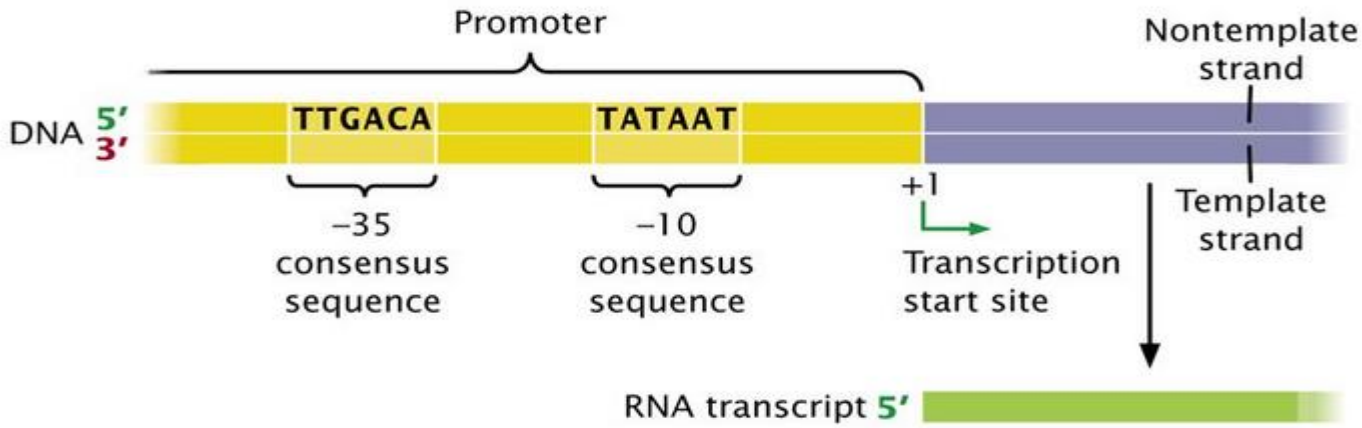
قدرت یک پروموتور:

۱- Affinity binding site

۲- Regulatory sites

۳- سرعت RNA elongation پلیمراز

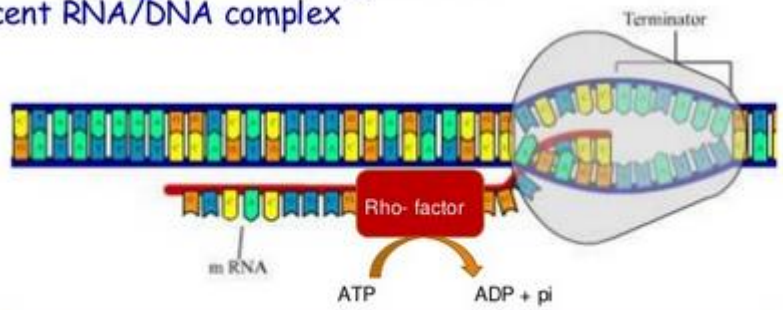
Upstream consensus sequences in bacterial promoters

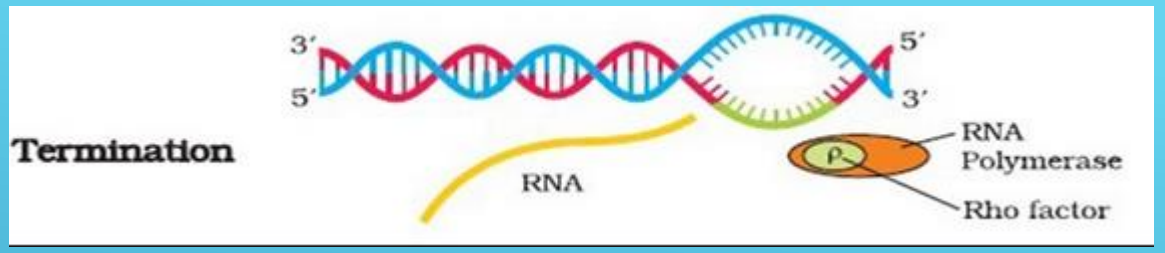


Termination

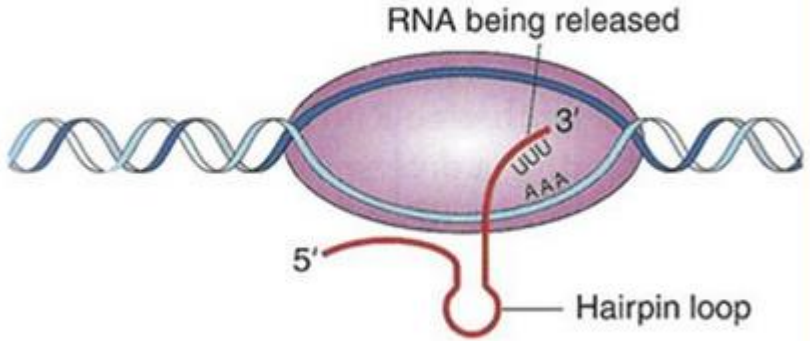
Rho factor is an ATP dependent RNA-DNA helicases

Recognizes and bind to the termination signals and disrupts the nascent RNA/DNA complex





Bacterial termination: intrinsic mechanism

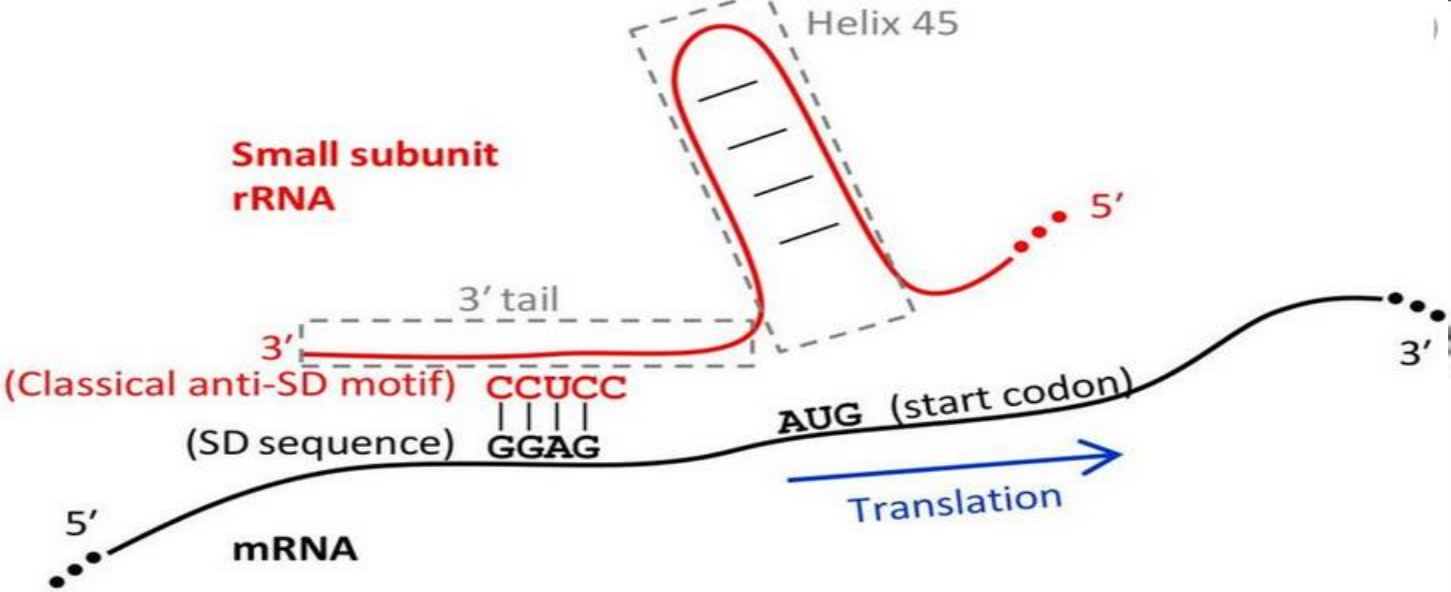


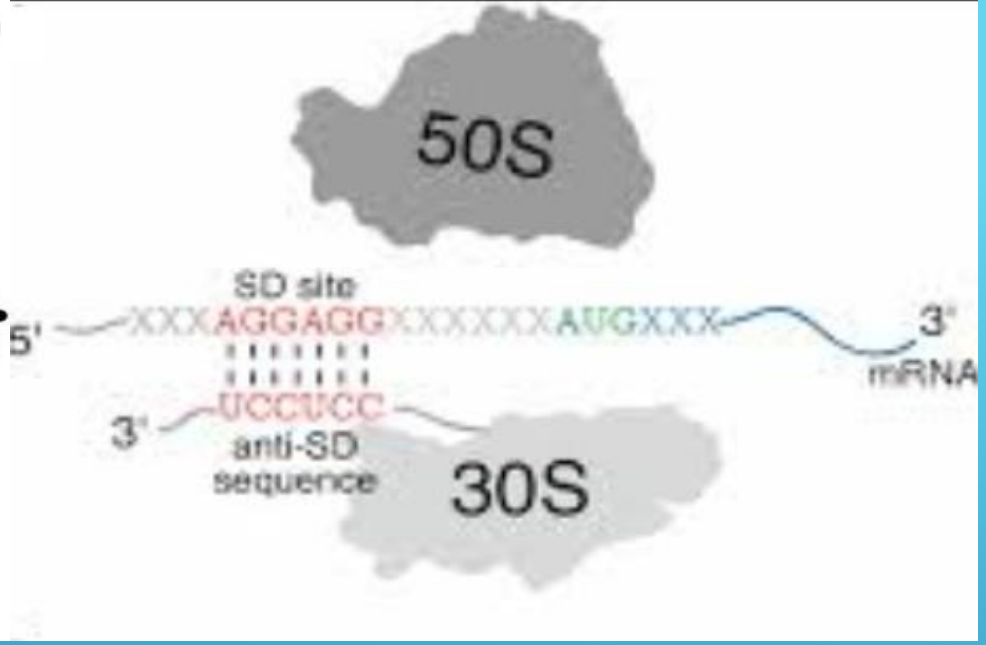
Prokaryotes:

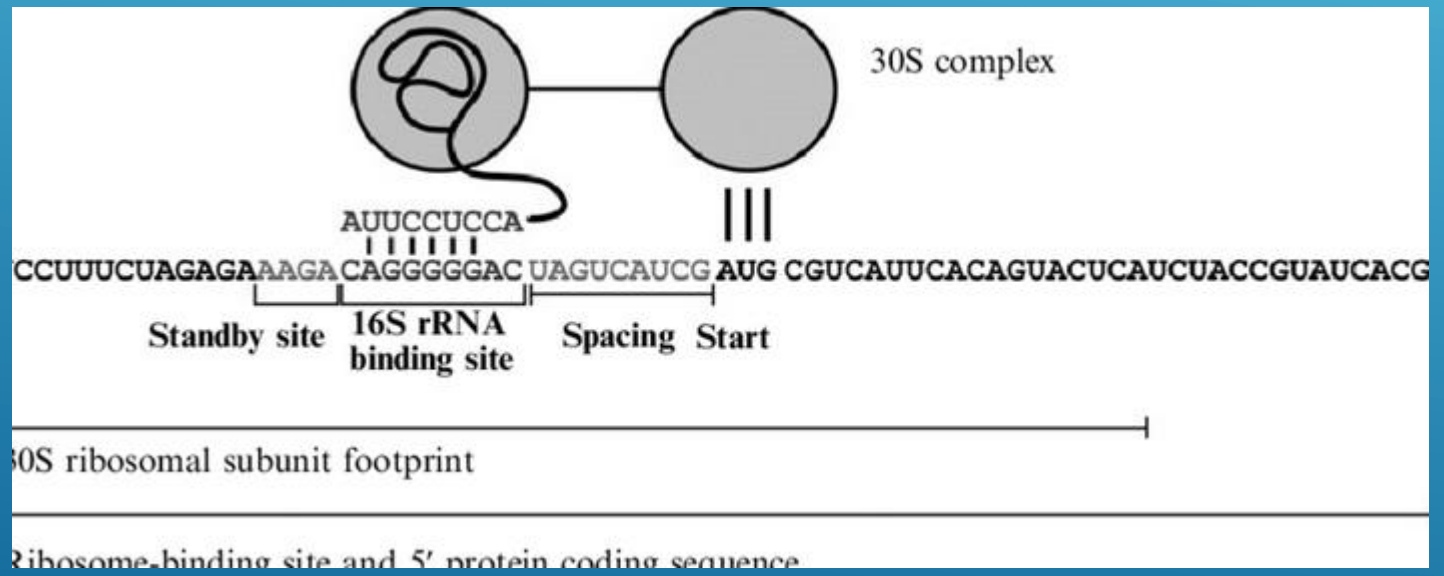
- mRNA transcript has a Shine-Dalgarno sequence
- rRNA on ribosome small subunit has a complementary section: anti Shine-Dalgarno sequence

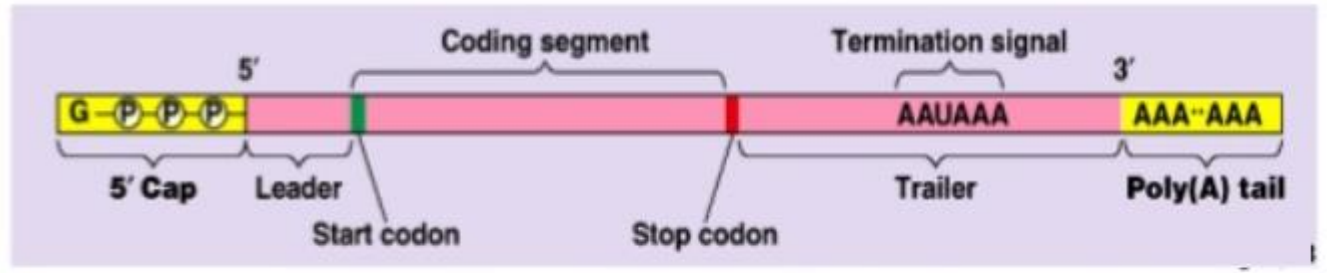
Eukaryotes

- Ribosome small subunit recognizes and bind to mRNA at 5' cap


Helix 45
Small subunit rRNA
5′
3′ tail
3′
(Classical anti-SD motif) CCUCC
(SD sequence) GGAG
AUG (start codon)
3′
5′
mRNA
Translation

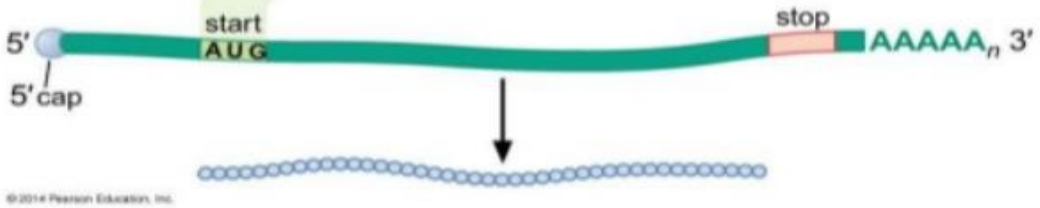

50S
SD site
5′
XXXAGGAGGXXXXXXAUGXXX
3′
mRNA
3′
UCCUCC
anti-SD sequence
30S


30S complex
AUUCCUCCA
CCUUUCUAGAGAAAGACAGGGGGACUAGUCAUCGAUG CGUCAUUCACAGUACUCAUCUACCGUAUCACG
Standby site
16S rRNA binding site
Spacing
Start
30S ribosomal subunit footprint
Ribosome-binding site and 5′ protein coding sequence



Kozak sequence

5′ NN $\frac{G}{A}$ NN AUG GNN 3′



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Organism(s)	Consensus Sequence*
Vertebrates	GCCRCCATGG
Terrestrial plants	AACAATGGC
Drosophila melanogaster (fruit fly)	CAAAATG
Saccharomyces cerevisiae (baker's yeast)	AAAAAAATGTCT
Dictyostelium discoideum (slime mold)	AAAAAAATGRNA
Plasmodium spp. (malarial protozoa)	TAAAAAATGAAN

*R = purine; N = any base.