Determining the function of YHR087W

Jaspreet Basutta
Nazde Edeer
Timothy Goh
Jen-Ai Lai

Introduction

- YHR087W is also known as *rtc3**
- Located on chromosome VIII
- Protein is found in nucleus and cytosol
- Known to be involved in RNA metabolism

**we will refer to YHR087W as RTC3

Amino acid sequence - domains

1 MSTVTKYFYKGENTDLIVFAASEELVDEYLKNPSIGKLSEVVELFEVFTPQDGRGAEGEL
61 GAASKAQVENEFGKGKKIEEVIDLILRNGKPNSTTSSLKTKGGNAGTKAYN*

1 MSTVTKYFYKGENTDLIVFAASEELVDEYLKNPSIGKLSEVVELFEVFTPQDGRGAEGEL
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61 GAASKAQVENEFGKGKKIEEVIDLILRNGKPNSTTSSLKTKGGNAGTKAYN*

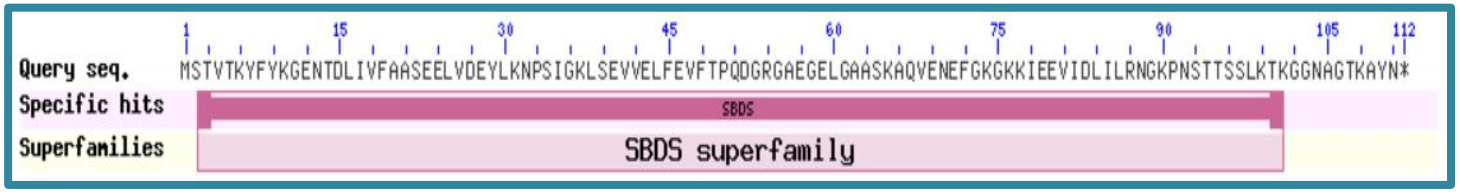
Sequence adapted from Uniprot (accession number: P38804)

- In **pink (3-98)**: Shwachman-Bodian-Diamond syndrome (SBDS) protein domain
- In **orange (1-109)**: FYSH domain - unknown function
- In **green (2-108)**: G3DSA:3.30.1250.10 domain - unknown function

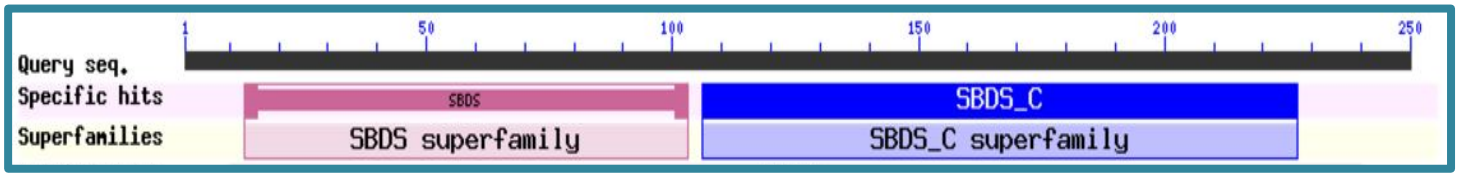
RTC3 & other proteins

RTC3 shares a **conserved domain** with the N-terminus of the human SBDS protein.

RTC3

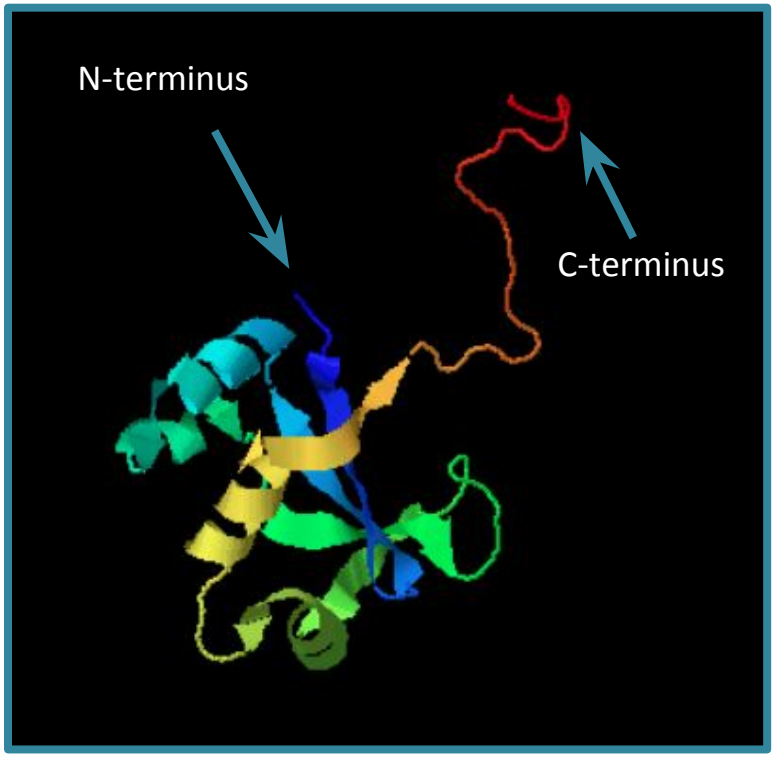


Human SBDS



Figures adapted from NCBI, amino acid sequences adapted from Uniprot (accession number P38804 & Q9Y3A9)

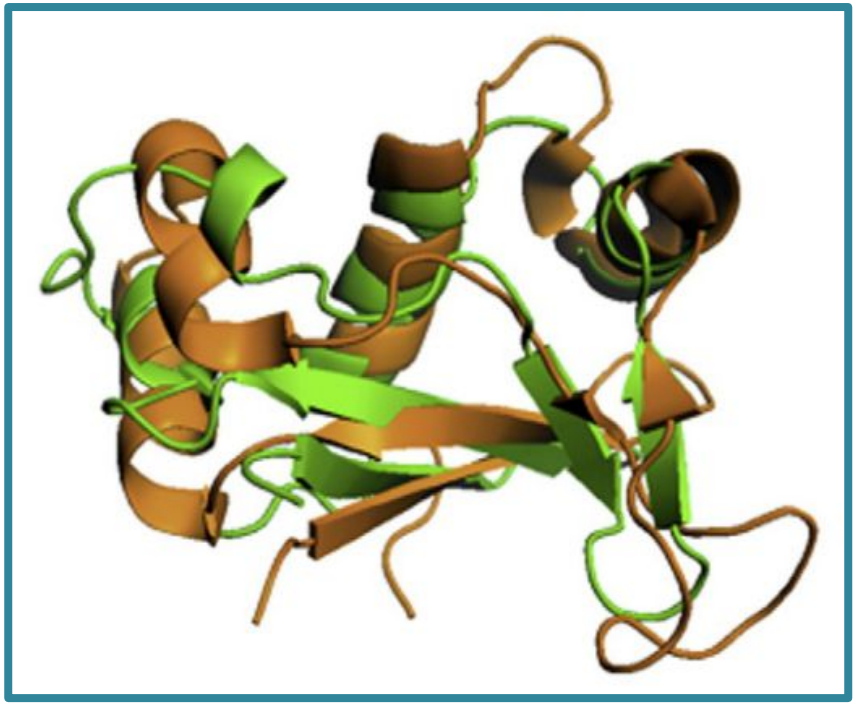
3D structure of RTC3



Structure generated with I-TASSER (ID: S261363)

- Four α-helices and four β-strands
- Sequential arrangement β1–β2–α1–α2–β3– β4–α3–α4
- C-terminus may bind another molecule

Comparison of RTC3 & human SBDS



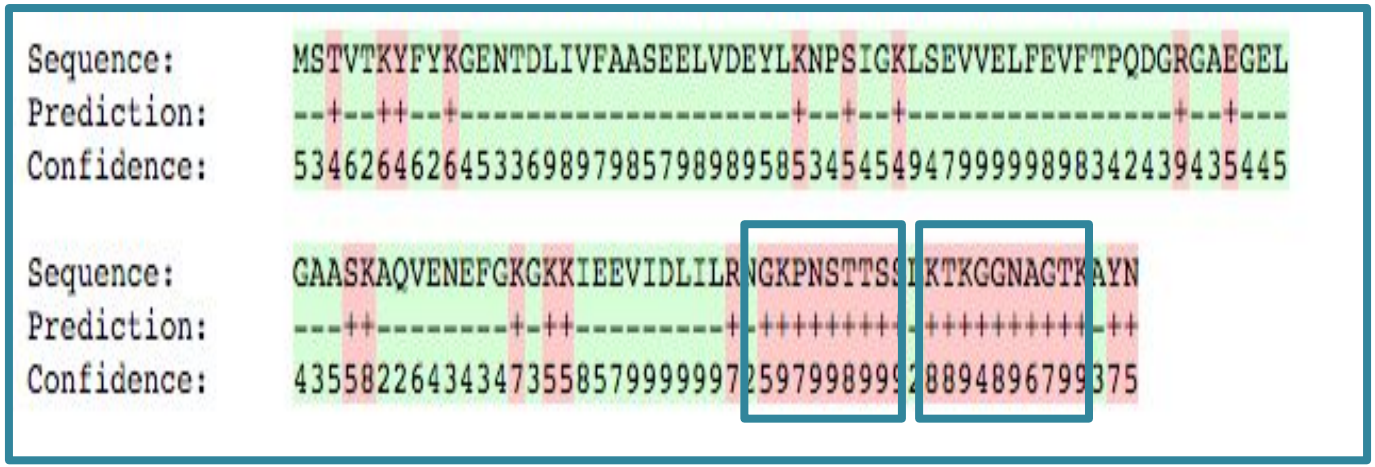
Adapted from the *Journal of Molecular Biology, 396,* 1058.

- Comparison of 3D structures
- Green: human SBDS N-terminus
- Brown: RTC3 protein

Human SBDS protein binds RNA

- Researchers used **HSQC spectroscopy** to measure H-N cross peak intensities, which indicate changes in bonds between atoms
- Ile29, Gln52, Phe57, Val58, Asn59, Ile72, and show **chemical changes in the presence of RNA**
- This is interpreted as **RNA-protein binding**
- These amino acids are located in the **N-terminus** of the SBDS protein

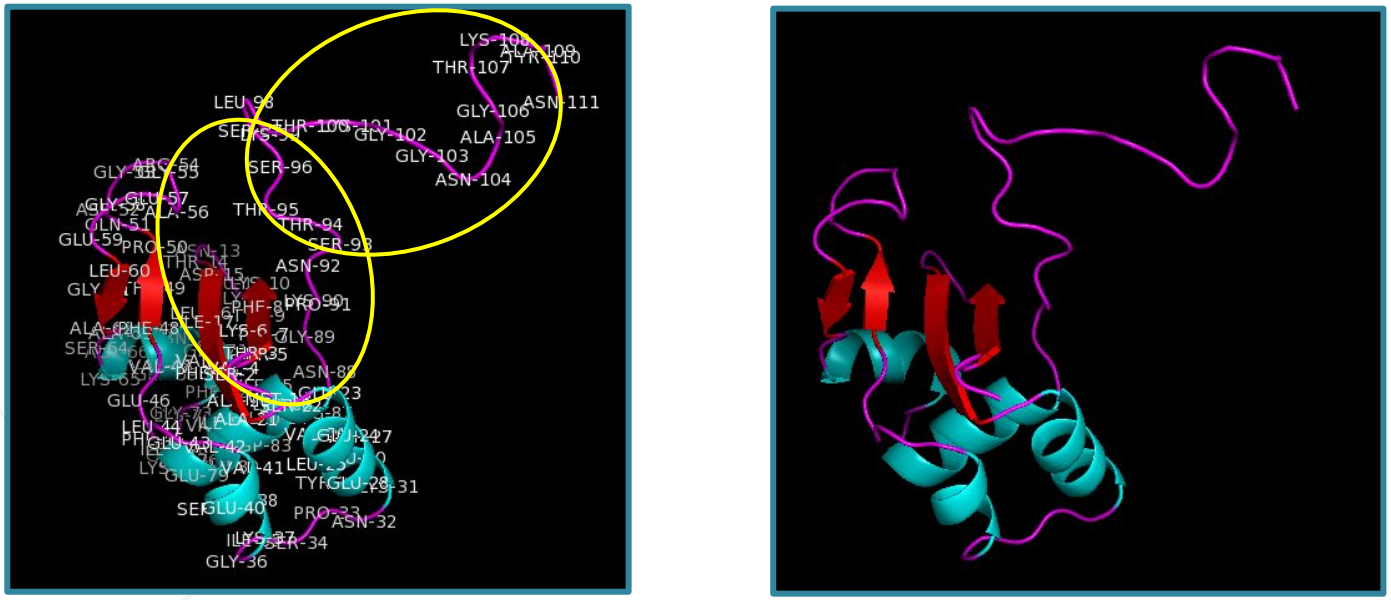
RTC3 has potential RNA binding sites



Predictions generated by BindN

- **Red** indicates amino acids that are predicted to bind RNA
- **Green** indicates amino acids that are predicted to NOT bind RNA
- Higher confidence scores indicate a higher certainty of the predicted interaction

RTC3 - predicted RNA binding sites

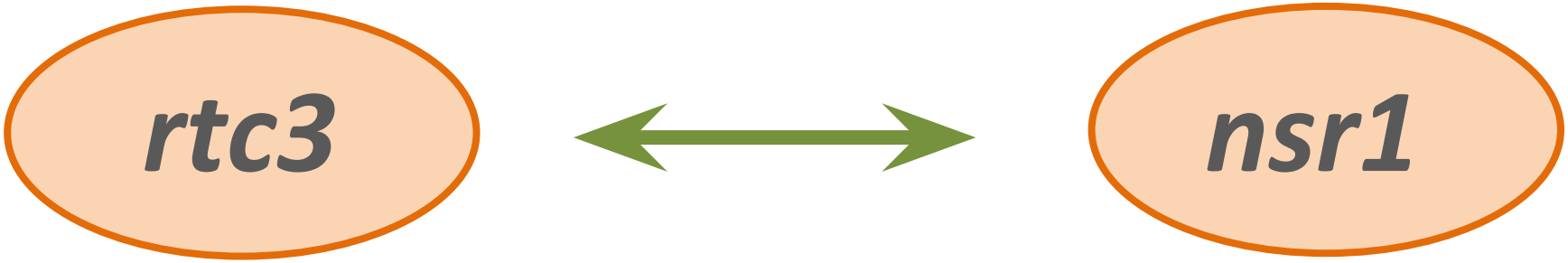


Structures generated by I-TASSER (ID: S261363), edited with PyMOL

- **Yellow** circles in Figure 1 indicate predicted RNA binding sites
- Binding site 1: ranges from Glycine-89 to Serine-97
- Binding site 2: ranges from Lysine-99 to Lysine-108
- Figure 2 shows the structural motifs of the gene - α-helices are in **blue** and β-strands are in **red**

rtc3 interactions

rtc3 interacts genetically with *nsr1*– another yeast gene



NSR1 | YGR159C, SHE5, L000001278

Nucleolar protein that binds nuclear localization sequences; required for pre-rRNA processing and ribosome biogenesis

UBI PHO SUMO

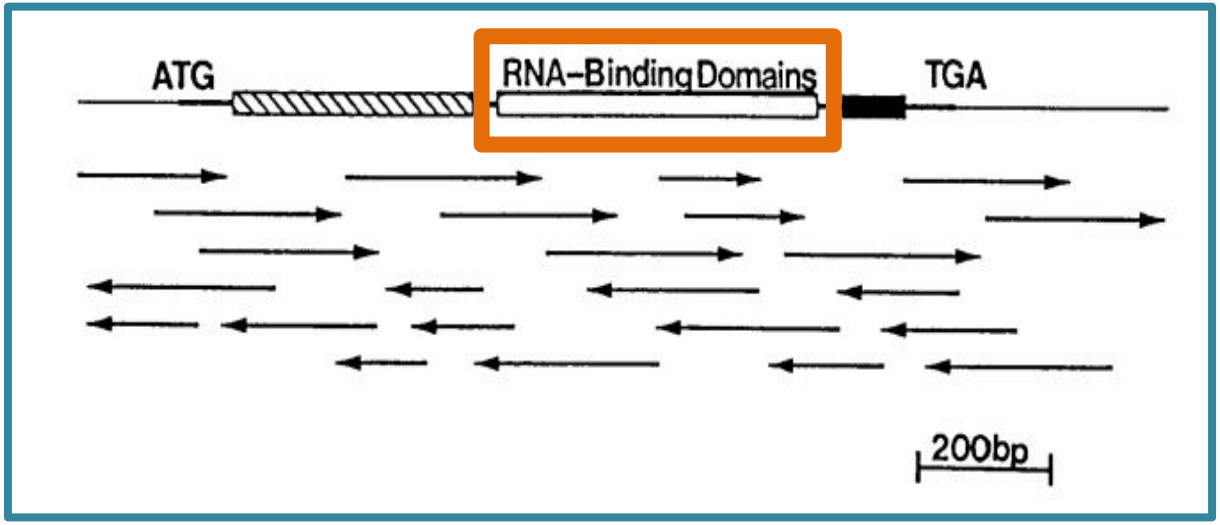
1 [details]

Experimental Evidence Code	Role	Dataset	Throughput	Curated By	Notes
Synthetic Lethality ⊞	HIT	Savchenko A (2005)	Low	BioGRID	

Interaction determined using BioGRID 3.4

What does the NSR1 protein do?

- NSR1 has **2 RNA recognition motifs**
- It interacts with initial **35S pre-rRNA** to facilitate **RNA processing**
- There is **synthetic lethality** between RTC3 and NSR1



Adapted from *The Journal of Cell Biology, 113*(1), 7

Synthetic lethality between RTC3 & NSR1

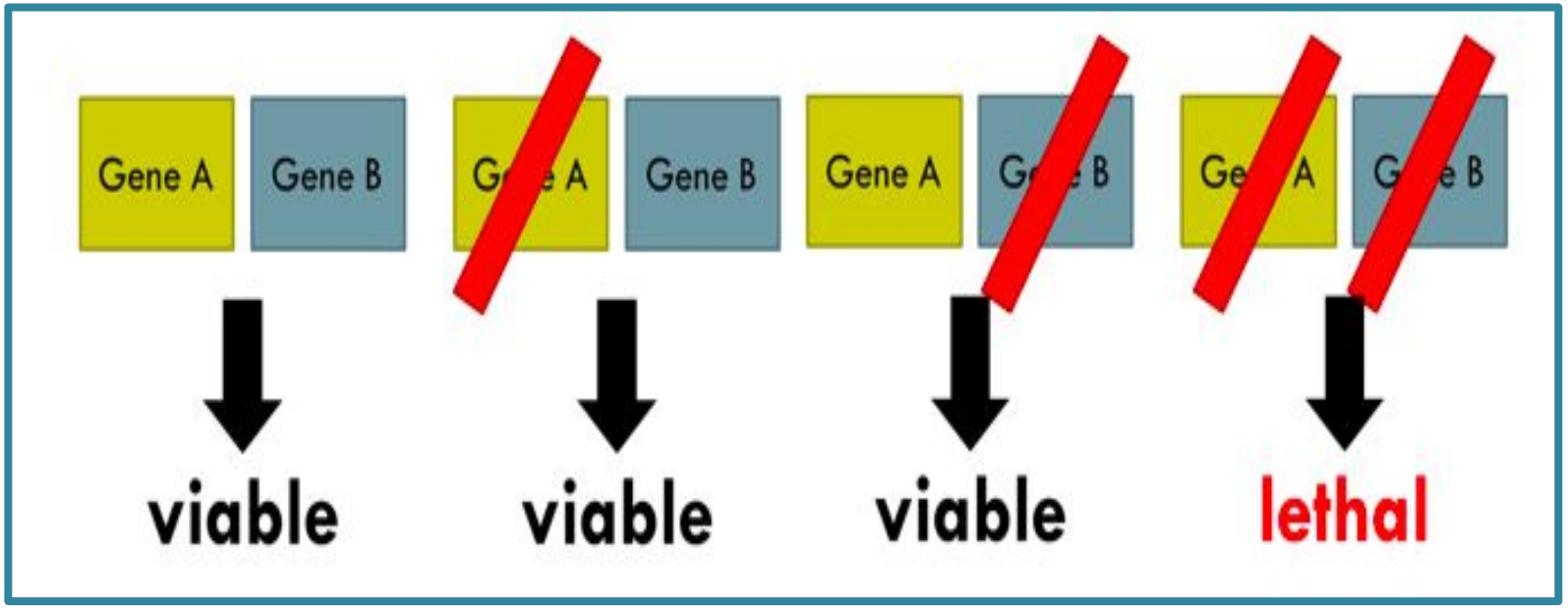


Figure adapted from https://en.wikipedia.org/wiki/Synthetic_lethality

Synthetic lethality indicates that **two genes may affect a single function or pathway**. Knockout of either gene does not cause cell death, but knockout of both genes does.

Hypothesis

RTC3 binds to 35S pre-rRNA through its RNA binding domain, and this interaction is required for 35S pre-rRNA processing.

Experiment 1

Aim:
Test whether RTC3 binds 35s pre-rRNA

Method:
Electrophoretic mobility shift assay (EMSA)

Experiment 1 - EMSA

- Detects **RNA protein interactions**
- Is non-denaturing to maintain non-covalent interactions
- **Naked RNA is more mobile** on a non-denaturing gel than RNA bound to a protein
- RNA bound to a protein results in an **upward shift on the gel**

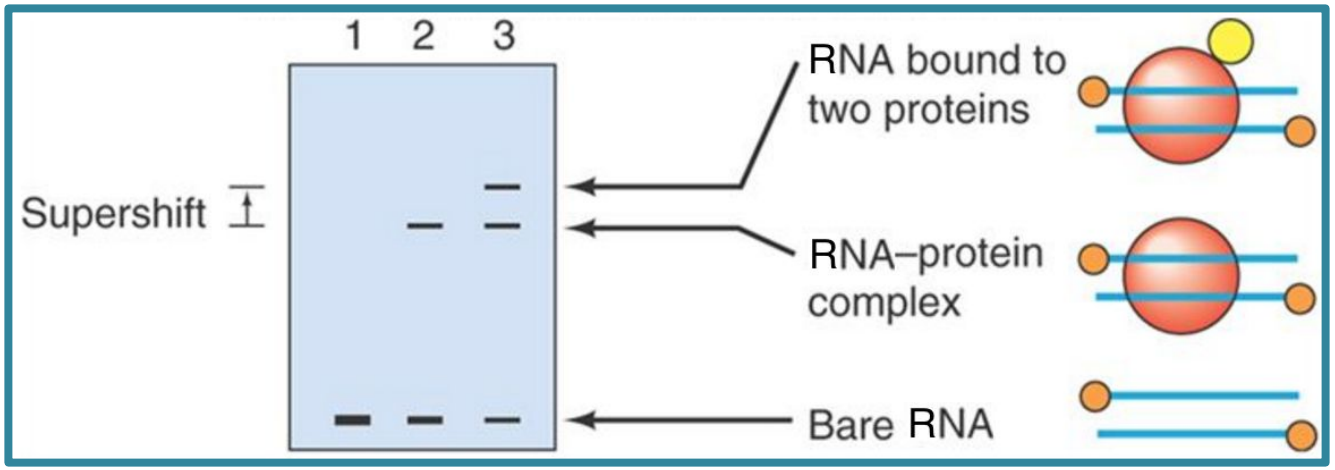
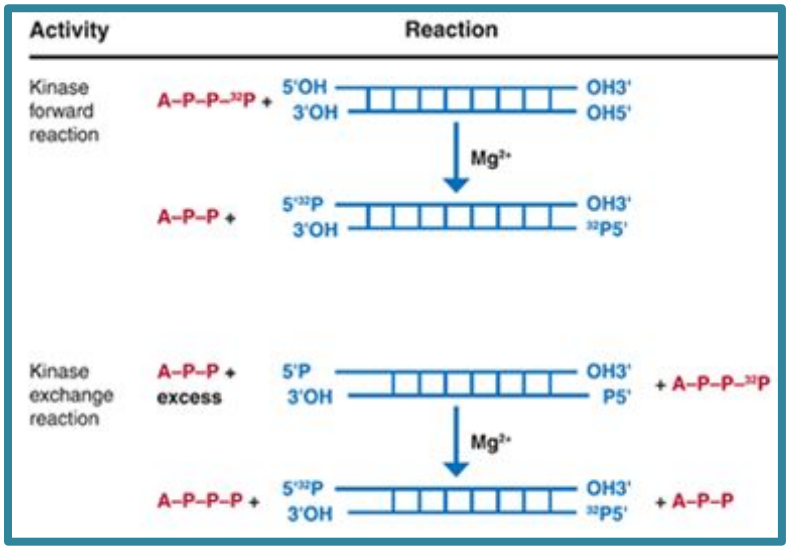


Figure adapted from Molecular Biology 4th Edition (McGraw Hill)

Experiment 1 - part 1

T4 polynucleotide kinase mechanism



Adapted from *Electrophoretic Mobility Shift Assays for RNA-Proteinhow to isolate protein in Complexes*. Retrieved from http://cshprotocols.cshlp.org/content/2014/4/pdb.prot080721.full

Part 1- Prepare 35S pre-rRNA for EMSA

Method:

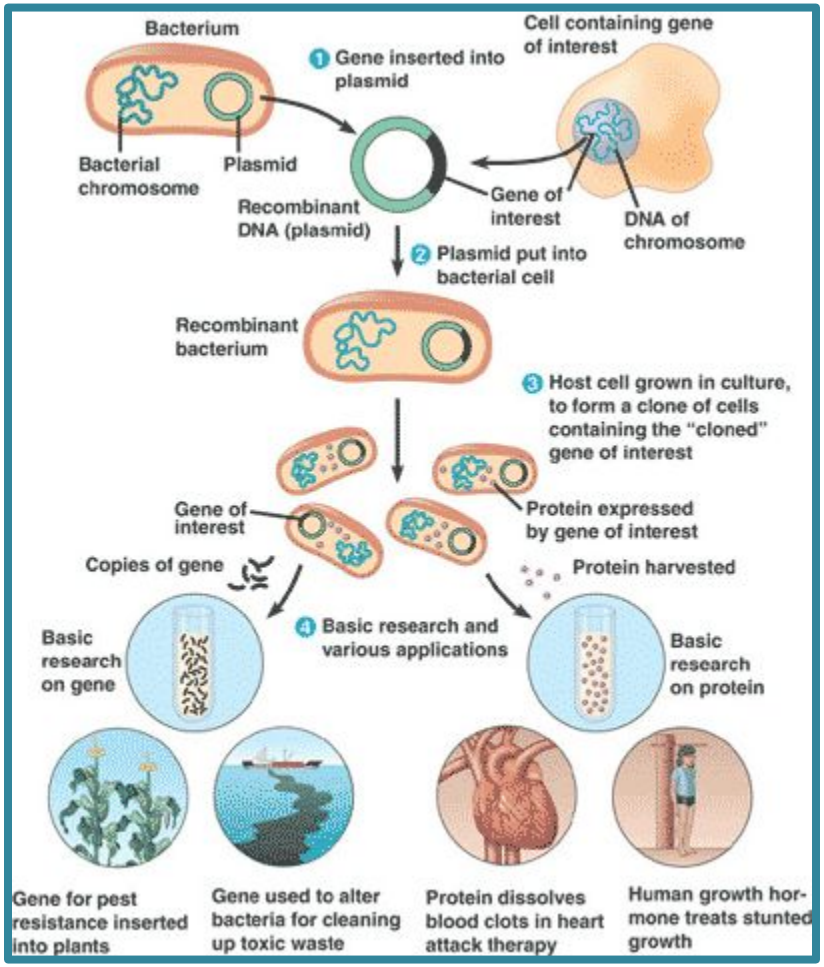
1) Generate RDN37-2 (35S pre-rRNA) DNA template using PCR

2) Use in vitro RNA transcription to generate & isolate 35S pre-rRNA

3) Use T4 polynucleotide kinase to label 5’ end of 35S pre-rRNA with 32-phosphate

4) Purify the 5’ labeled 35S pre-rRNA and check probe activity with scintillation counter

Experiment 1 - part 2



Adapted from https://www.quia.com/jg/1283042list.html

Part 2 - Clone *RTC3* into E.Coli, amplify & isolate RTC3

Method:

1) Use bacterial transformation to insert tagged *RTC3* into E. Coli cells.

2) Select for colonies with recombinant plasmid, allow them to generate RTC3.

3) Isolate & purify RTC3.

Experiment 1 - part 3

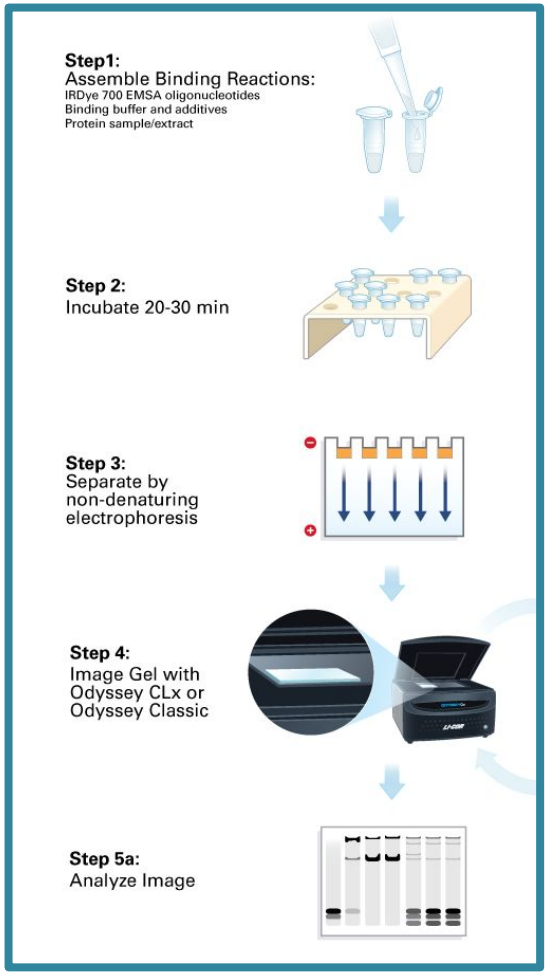


Figure retrieved from https://www.licor.com/bio/applications/emsa/workflow.html

Part 3 - EMSA

Method:

1) Combine P-32 labeled 35S pre-rRNA and purified RTC3 .

2) Visualize reaction products by running them on a non-denaturing polyacrylamide gel.

3) Image gel using a phosphorimager cassette or X-ray film.

Experiment 1 - predicted results

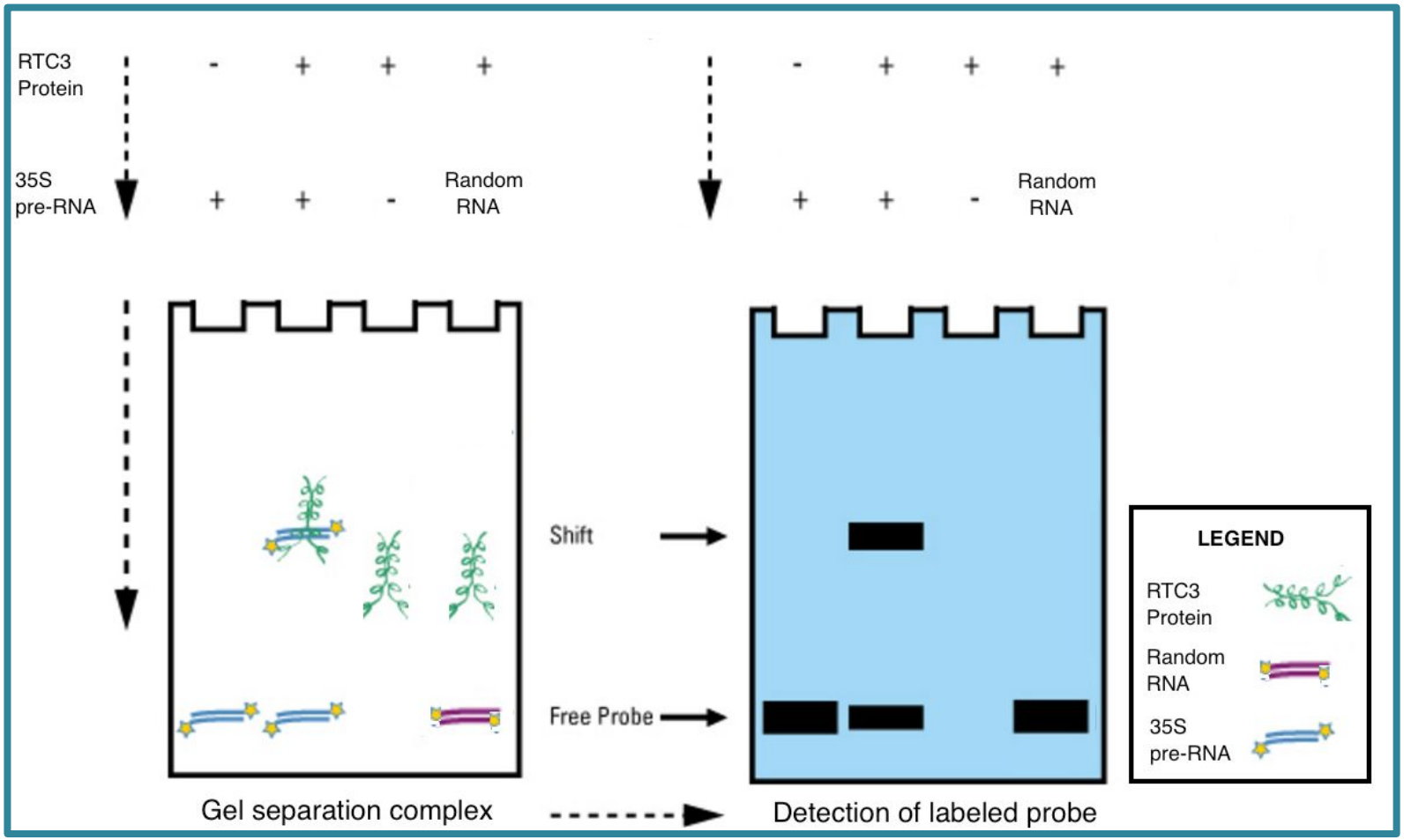


Figure adapted from ThermoFisher Scientific Gel Shift Assay

Experiment 1 - summary

If RTC3 binds 35S pre-rRNA:

- We can use **EMSA** to look for shifts in a gel
- 35S pre-rRNA + RTC3 will be **less mobile** than unbound rRNA in a gel
- We expect an **upward shift** on the gel in the lane with RTC3 + 35S pre-rRNA when compared to the free 35S pre-rRNA
- We expect **no shift** in the lane with RTC3 & RTC3 + random RNA

Experiment 2

Aim:
Test whether RTC3 is required for 35s pre-rRNA processing

Method:
Reverse transcriptase quantitative PCR (RT-qPCR)

Experiment 2 - RT-qPCR

Reverse transcription is used to make complementary DNA from RNA.

Quantitative PCR uses fluorescently labelled DNA to generate amplification curves and quantitate gene products.

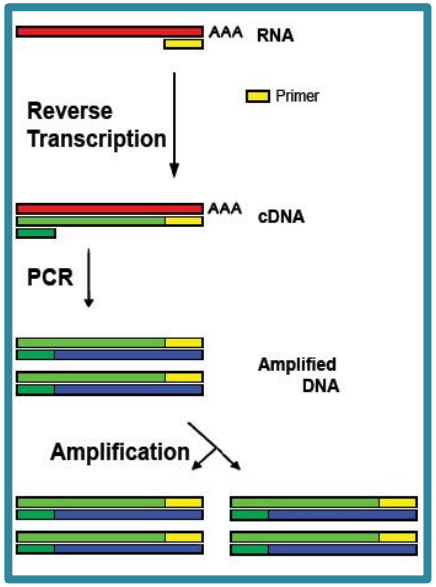
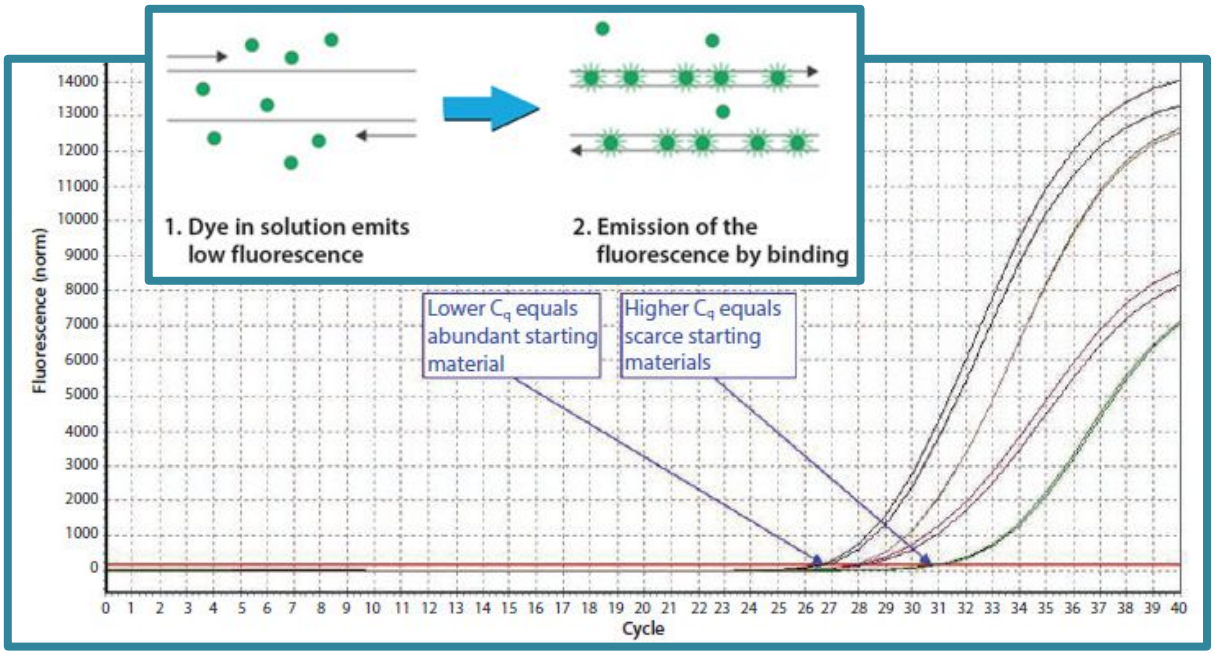


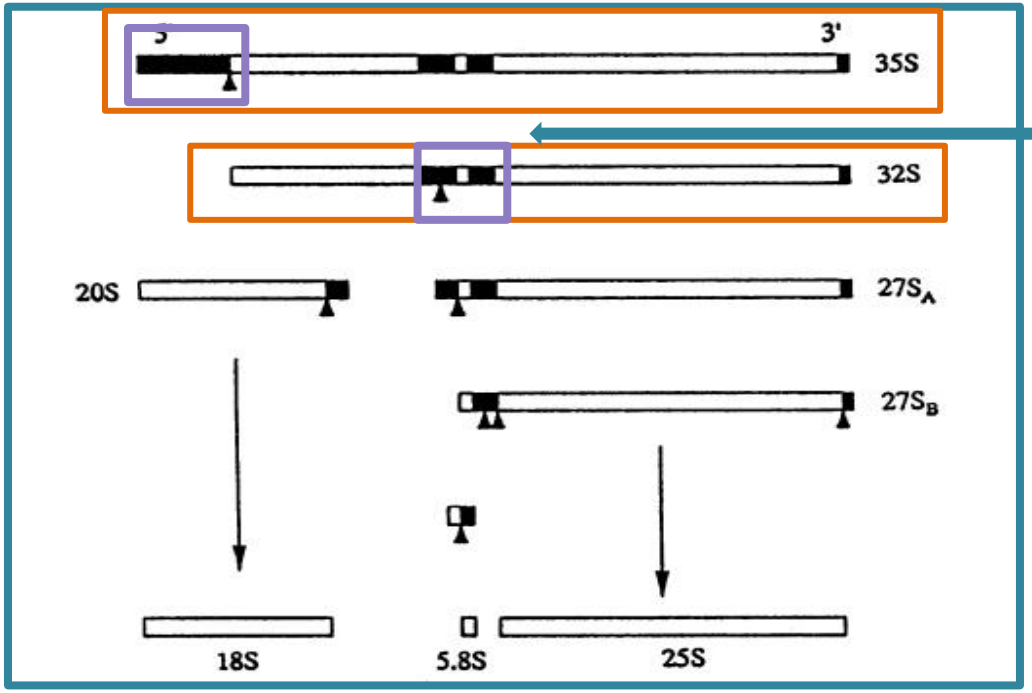
Figure adapted from Wikipedia (Reverse transcription polymerase chain reaction)



Adapted from http://www.sigmaaldrich.com/technical-documents/protocols/biology/sybr-green-qpcr.html

Adapted from http://www.sigmaaldrich.com/technical-documents/articles/biology/quantitative-pcr.html

Experiment 2 - rationale

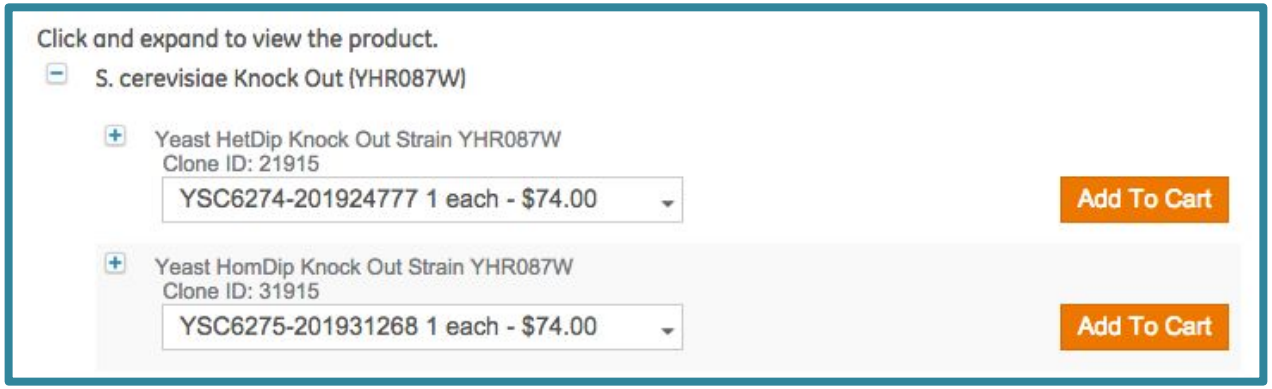


35S ⟶ 32S

Adapted from the *Journal of Molecular and Cell Biology*, *12*(9), 3866.

- RTC3 may play a **similar role to NSR1** in 35S pre-rRNA processing
- Cells lacking NSR1 have **increased 35S** and **decreased 32S** pre-rRNA levels

Experiment 2 - part 1



Adapted from http://dharmacon.gelifesciences.com/non-mammalian-cdna-and-orf/yeast-knockout-collection/?term=RTC3&sourceId=EG/856487&productId=FE10A61B-D7FD-4FF6-93A0-944163AF627A

Part 1- Obtain *RTC3* mutant *S. Cerevisiae* & extract RNA.

Method:

1) Order knockout mutant *RTC3* in *S. Cerevisiae*.

2) Confirm mutation.

2) Grow mutant and wild-type (WT) yeast to use as a control.

3) Extract RNA using an extraction kit.

Experiment 2 - part 2

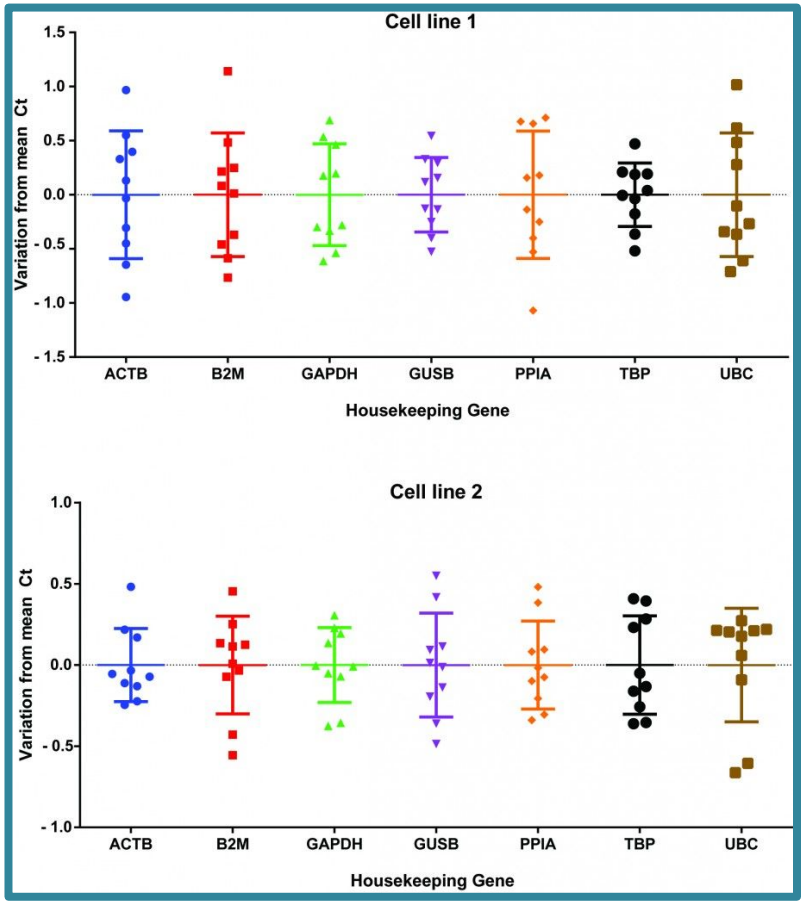
Part 2 - Choose a reference gene for RT-qPCR

Method:

1) Choose a selection of genes that are known to not vary in our experimental conditions.

2) Run RT-qPCR for these genes.

3) Select the one that varies the least between mutant & WT cells as your reference gene.



Adapted from Keeping On Top of Housekeeping Genes - Bitesize Bio.

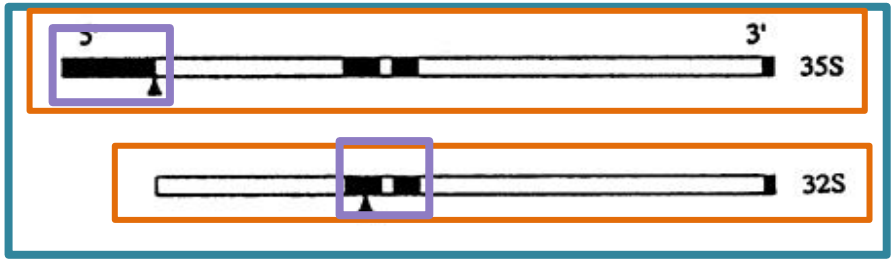
Experiment 2 - part 3

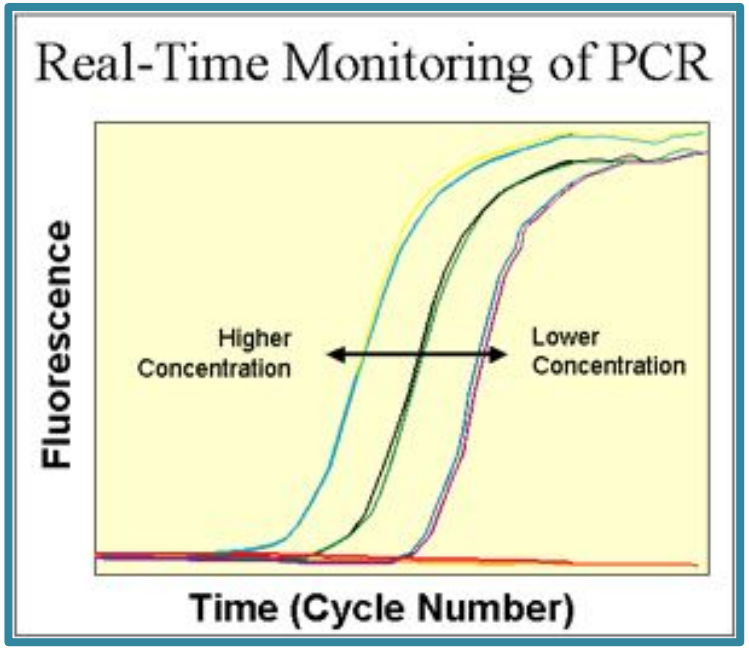
Part 3 - RT-qPCR
Method:

1) Order primers specific for 35s & 32s rRNA.

2) Use RT-qPCR to generate amplification curves for reference mRNA, 35S, 32S rRNA for WT & mutant cells.

3) Graph RT-qPCR data to look for relative changes in cDNA concentrations between WT & mutant cells.



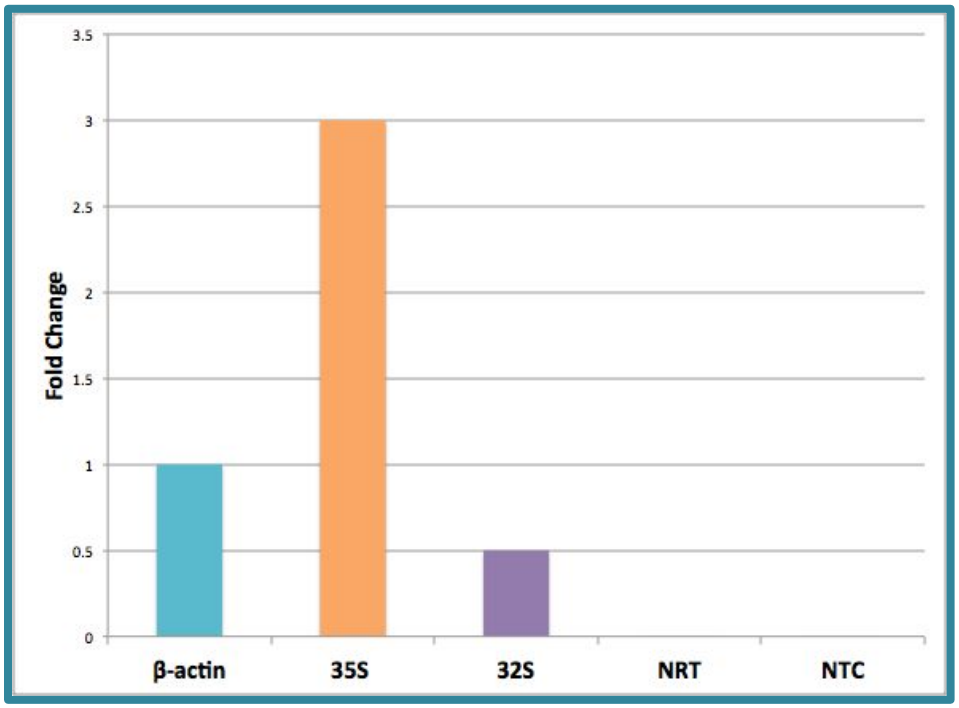


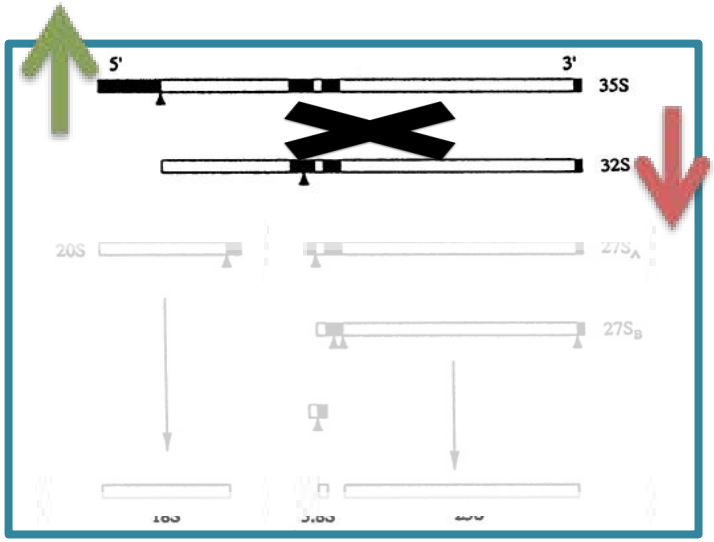
Adapted from https://dna.utah.edu/LightCycler/Top_LightCycler.html

Experiment 2 - predicted results

PREDICTION → build up of 35S pre-rRNA & decrease of 32S pre-rRNA

Δ(ΔCt) - Comparison of rRNA and reference gene mRNA levels in WT and mutant cells





How to interpret the data:

1 = no change between WT and mutants

>1 = increase in mutant cells

<1 = decrease in mutant cells

Experiment 2 - summary

If RTC3 is required for 35s pre-rRNA processing:

- Use a knockout mutant to disrupt *rtc3* gene function.
- With the use of a reference gene, **RT-qPCR** will allow us to quantify relative changes in cDNA levels.
- When 35S pre-rRNA processing is inhibited, we expect an increase in **35S pre-rRNA and a decrease in 32S pre-rRNA.**

Further research & significance

Further research:

- Determine which specific amino acids bind 35S pre-rRNA.
- Explore the impact on 40S subunit ribosome biogenesis.

Significance:

- Shed light on which proteins are functionally redundant with NSR1.
- Further our understanding of pre-rRNA processing.
- Help determine the molecular mechanisms responsible for Schwachman-Diamond syndrome.

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References

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