Leveraging the Power of In Vitro Transcription:
An Exploration of Applications, Challenges and Solutions

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Lucigen
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Agenda

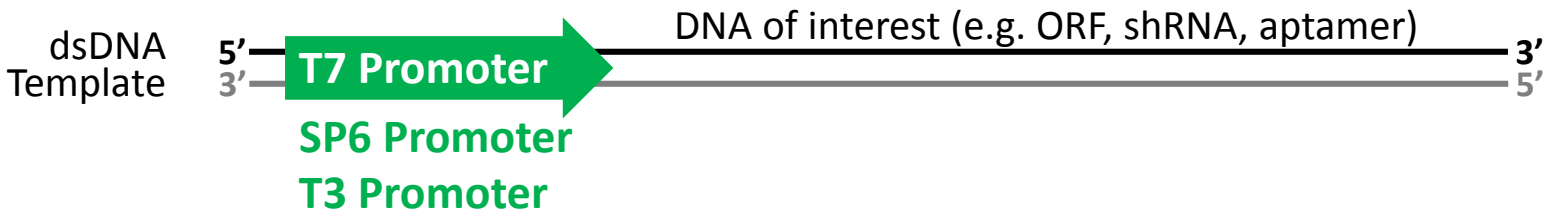
Leveraging In Vitro Transcription for Your Success

- Overview of in vitro transcription (IVT) technology
- Applications of IVT-produced RNA
  - Overview of each type of application
  - Examples of IVT use for each application with data from the literature
- Key challenges of IVT and working with RNA
- Solutions to those challenges
  - AmpliScribe™ T7-Flash Transcription Kit
  - DuraScribe® T7 Transcription Kit

In Vitro Transcription (IVT)

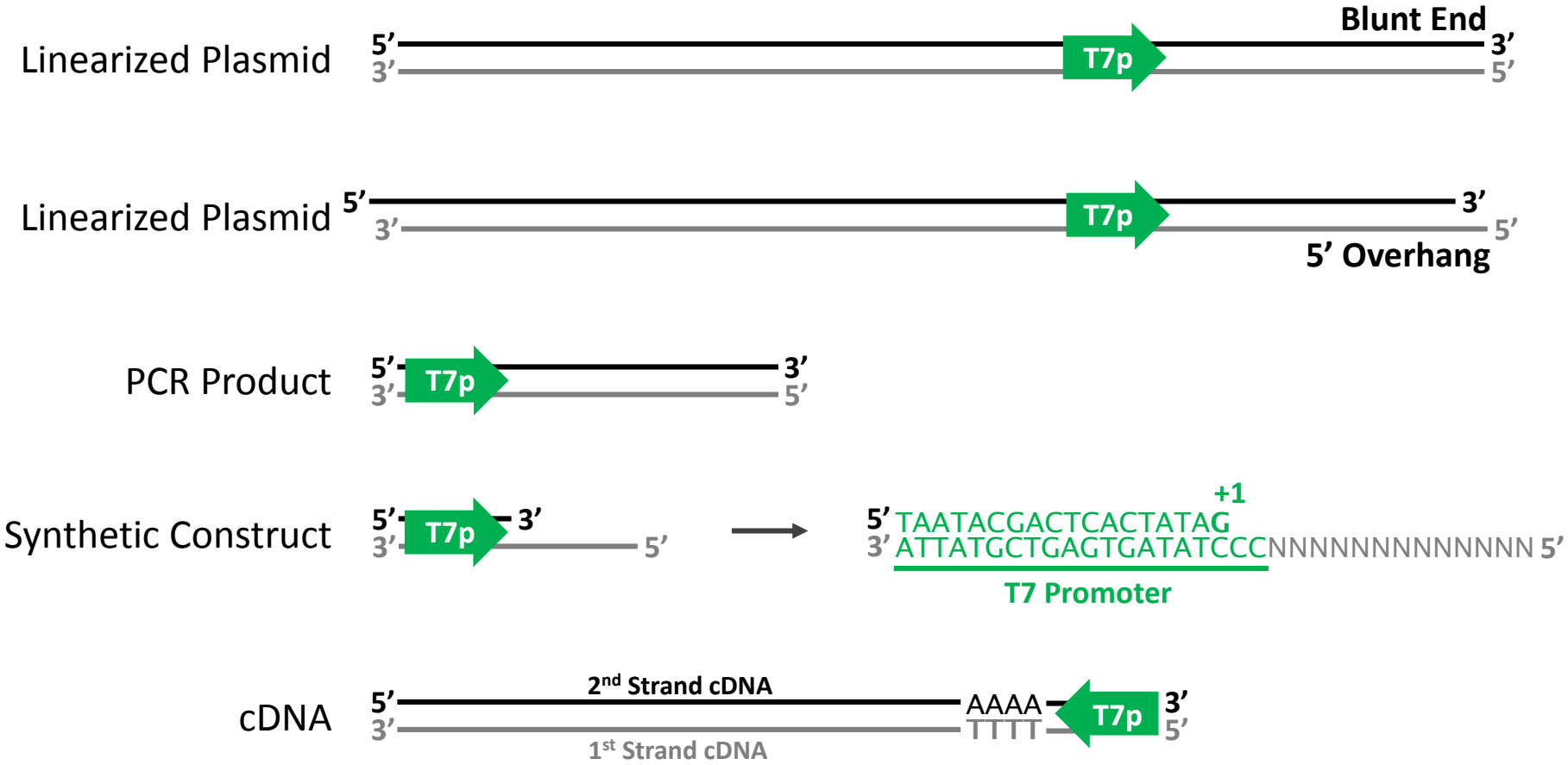
Transcription Generally Starts with a dsDNA Template

In Vitro Transcription:
Production of RNA in a tube (e.g. microfuge tube) using an RNA polymerase (usually phage derived), ribonucleotides and appropriate buffer conditions.



IVT Template Options

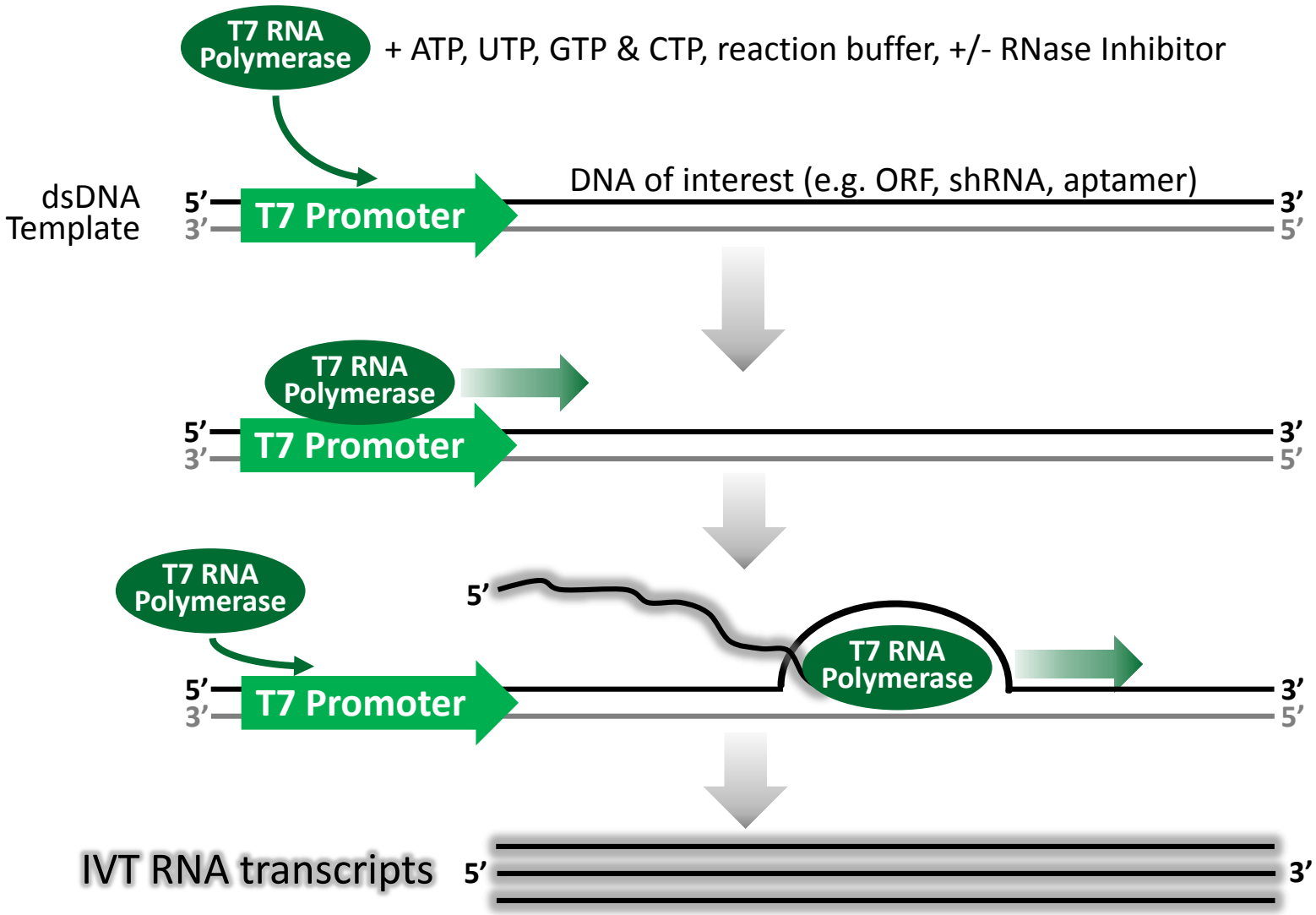
Multiple Forms of DNA Make Acceptable IVT Templates



Caution: 3’ overhangs may lead to the production of spurious RNA transcripts

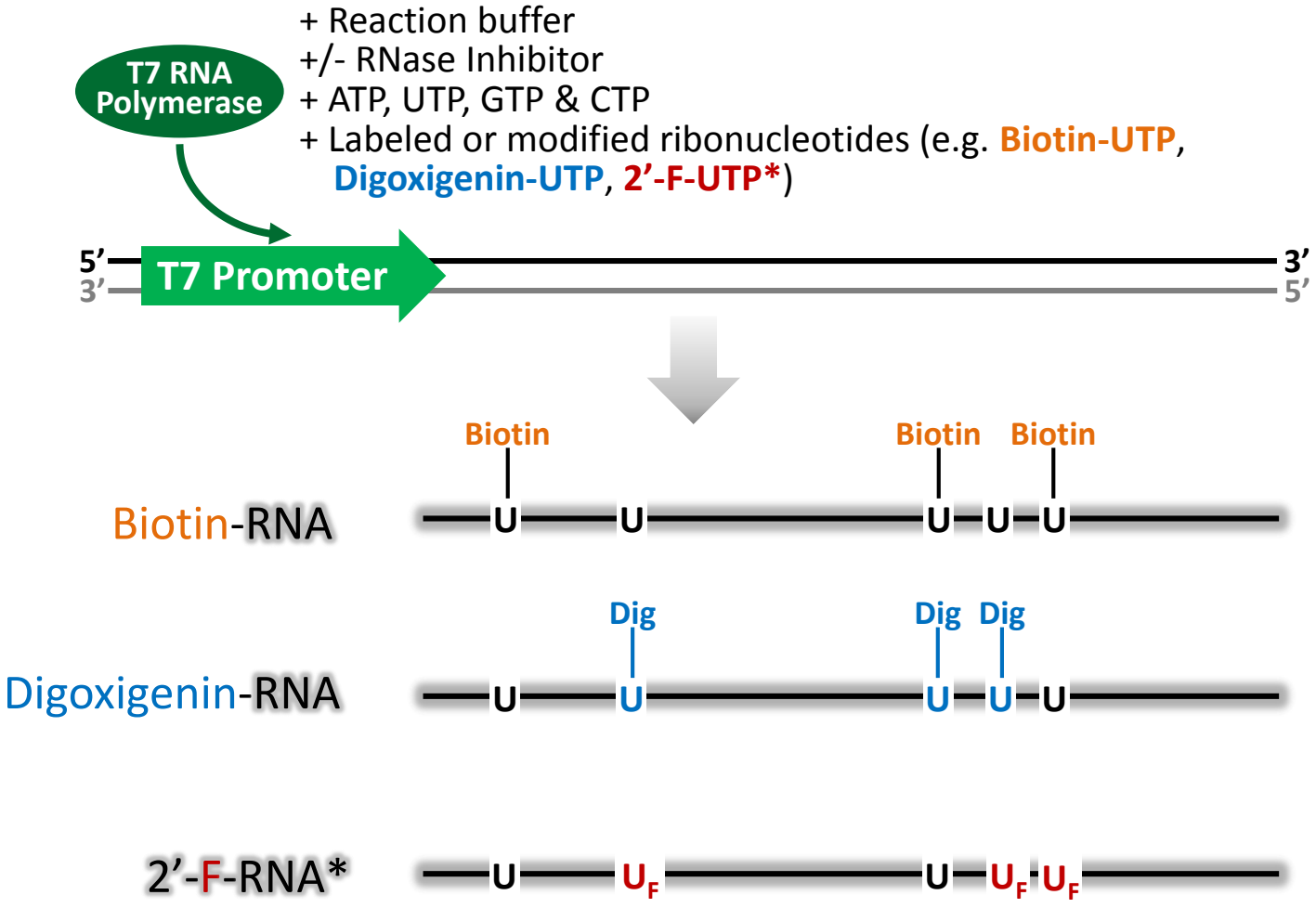
IVT Reactions

Production of Large Amounts of RNA from ~1 µg of DNA



Production of Modified or Labeled IVT RNA

Modified or Labeled NTPs are Effectively Incorporated



* Incorporation of 2'-F-UTP and 2'-F-CTP requires a mutated T7 RNA Polymerase

Note: It is also possible to incorporate radiolabeled ribonucleotides

Uses/Applications of IVT RNA

IVT RNA Makes Many Experiments Possible

- ✓ Viral RNA synthesis and transfection to start replication
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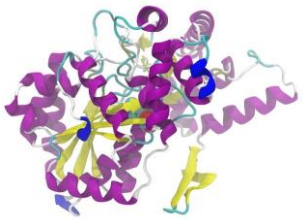

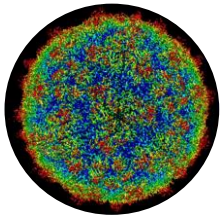

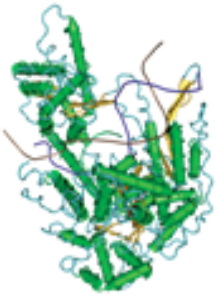

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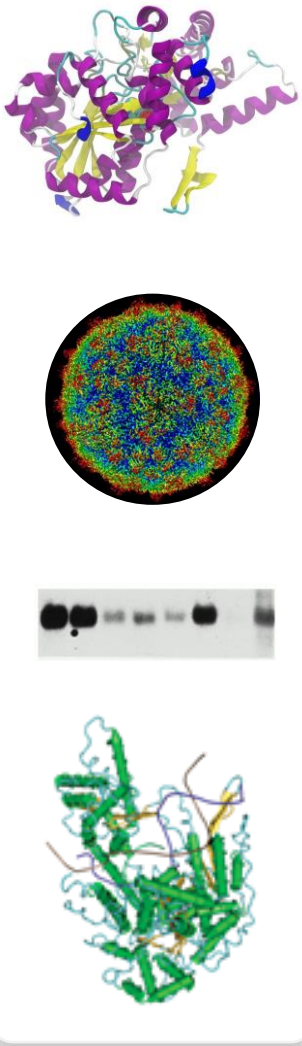
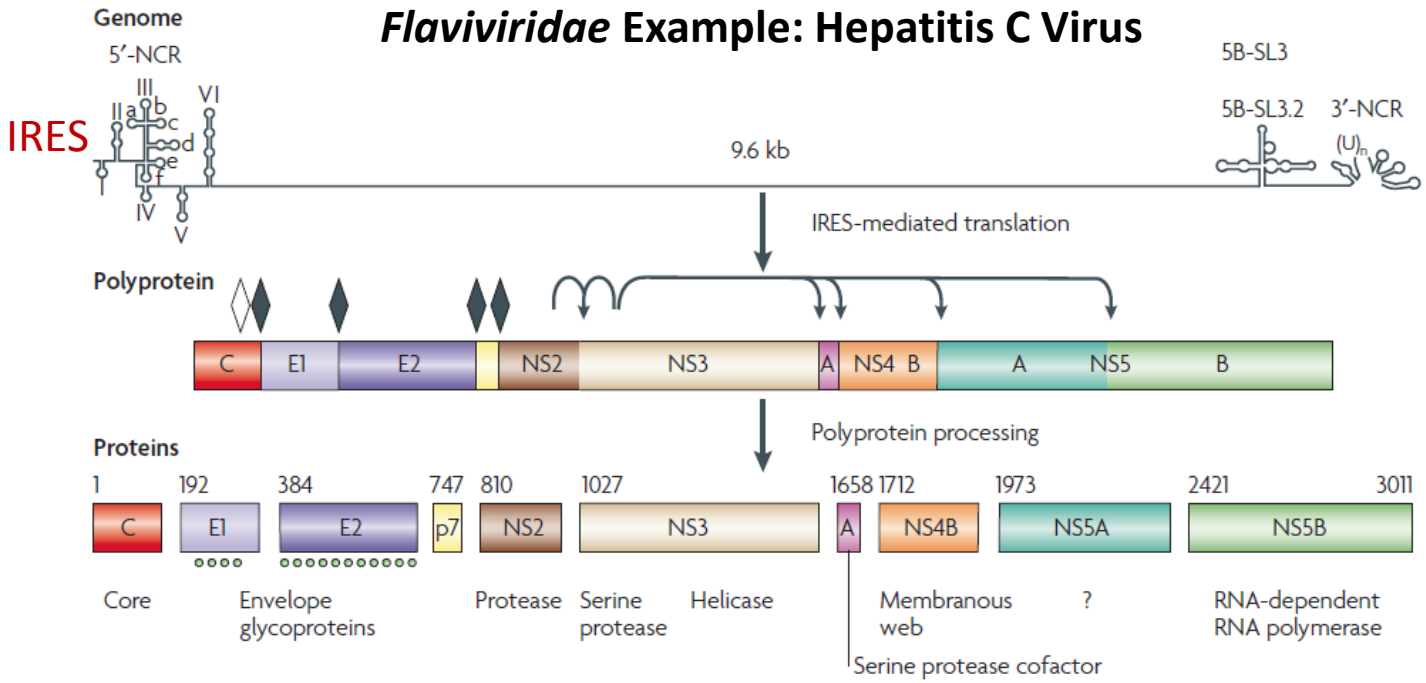


Flaviviradae Family Viruses

Great Models for All Things Related to IVT RNA



***Flaviviridae* Example: Hepatitis C Virus**

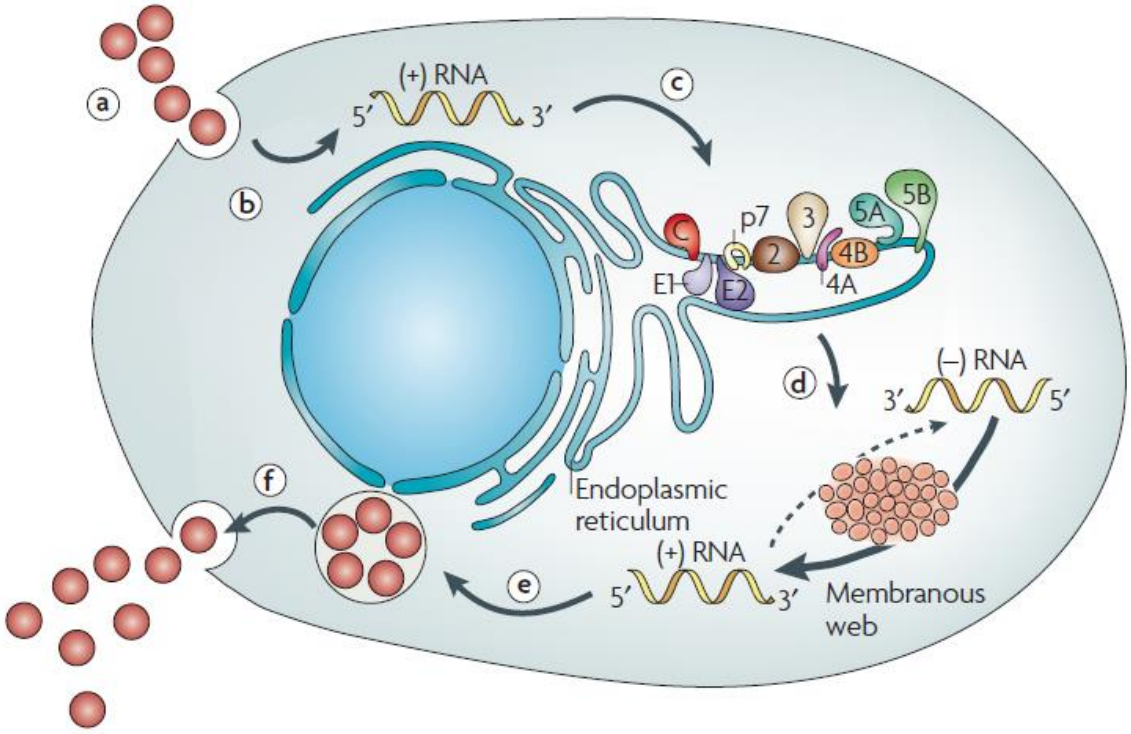
From: Moradpour, Penin and Rice (2007) Replication of Hepatitis C virus. *Nature Reviews | Microbiology* **5**:453-463

Hepatitis C Virus

- 9.6 kb +strand RNA genome – *Flaviviridae* family (e.g. Yellow fever, Dengue)
- After cellular entry, cell translates genomic RNA into a polyprotein using IRES to initiate
- Polyprotein is cleaved by **cellular protease (diamonds)** and **viral proteases (arrows)**
  - Proteolysis occurs co- and post-translationally

Hepatitis C Virus Replication Cycle

Replication Starts with Translation of the Incoming Genome



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a) Cell binding and entry

b) Uncoating and genome release

c) IRES-mediated translation and polyprotein processing

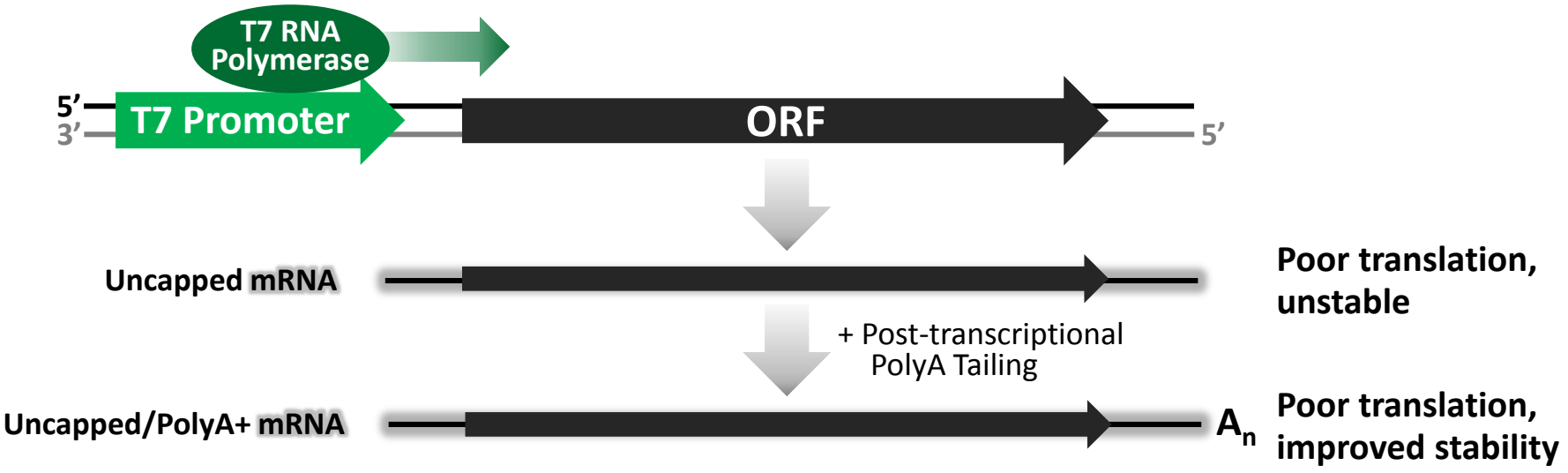
d) RNA genome replication (+RNA) → (-) RNA → (+) RNA

e) Packaging and assembly

f) Maturation and release

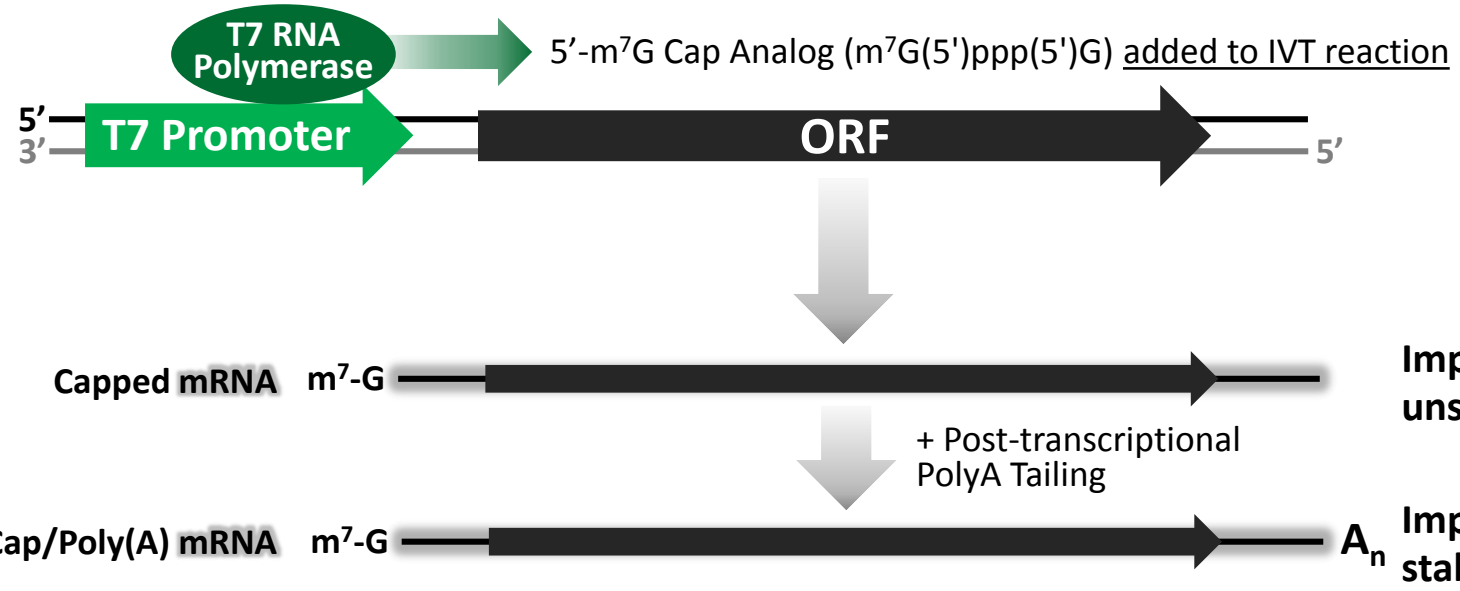
Translation of IVT RNA

Uncapped IVT RNAs are Poorly Translated

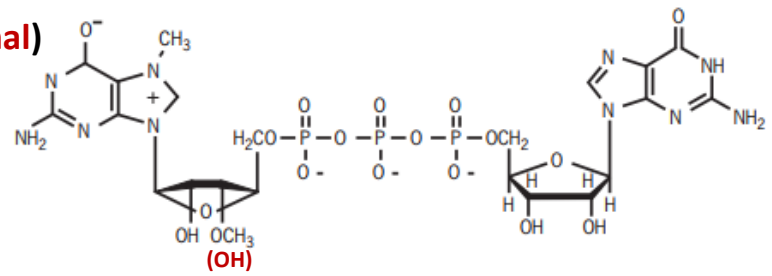


Translation of IVT RNA

Addition of a m^7-G Cap Analog Improves Translation



Cap Analogs (ARCA, Normal)

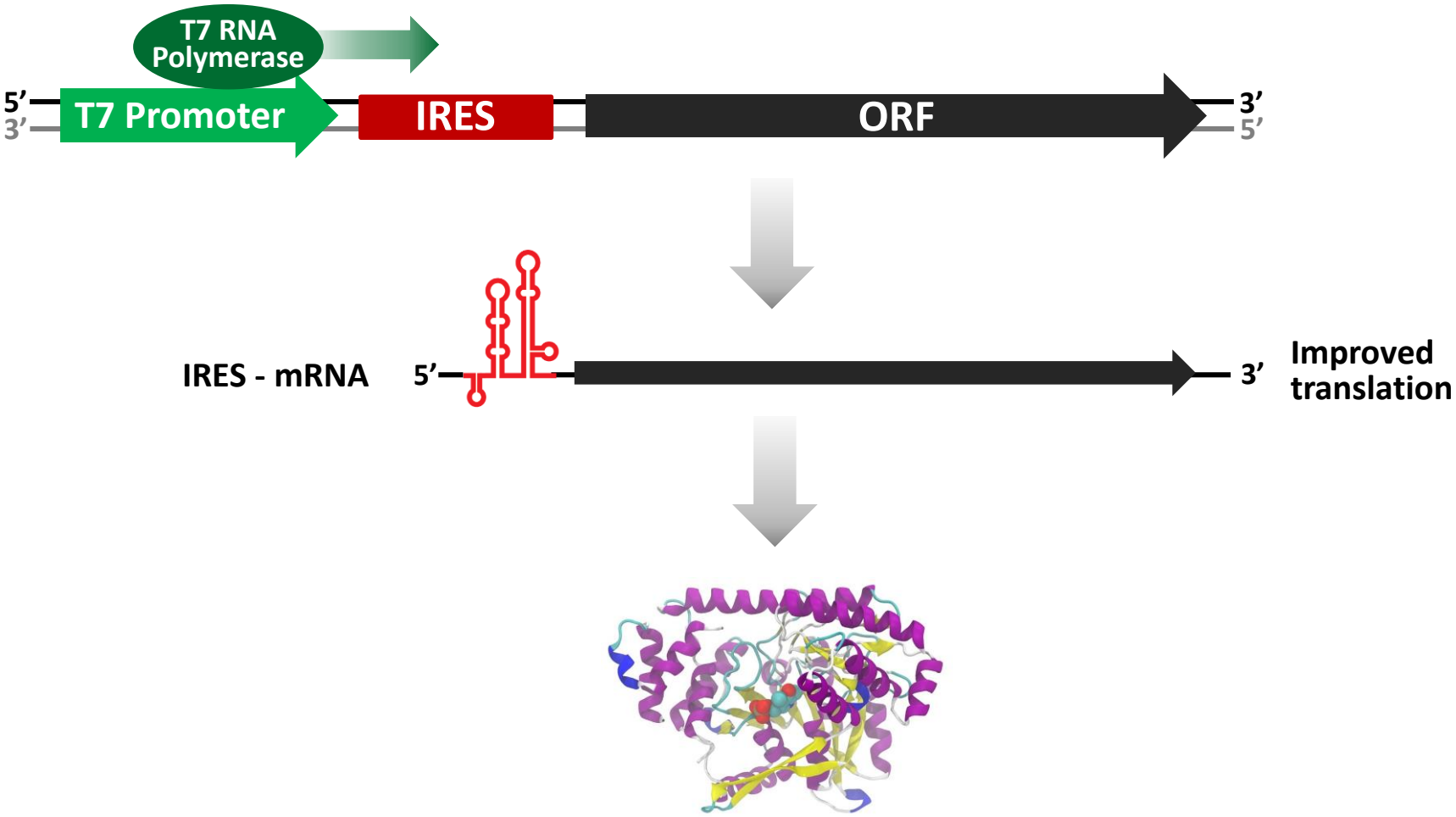


Note: 5' Caps can also be added post-transcriptionally

Translation of IVT RNA

Viral IRES Enhance Translation in the Absence of a Cap

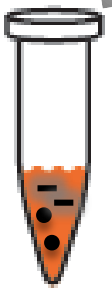
IRES = **I**nternal **R**ibosome **E**ntry **S**ite



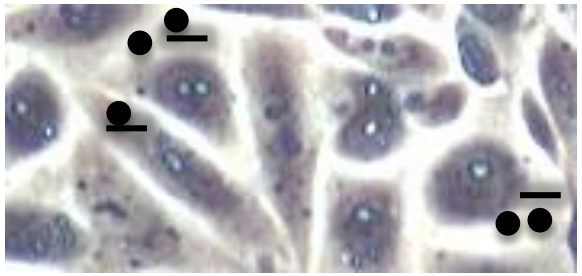
Translation of IVT RNA

IVT mRNA can be Translated In Vivo and In Vitro

✓ Cell-free protein expression
- Rabbit reticulocyte extracts
- Wheat germ extracts
- HeLa extracts



✓ Transfection into cultured cells
✓ Microinjection (e.g. oocytes)



Benefits
- Speed
- No cellular toxicity
- Better solubility

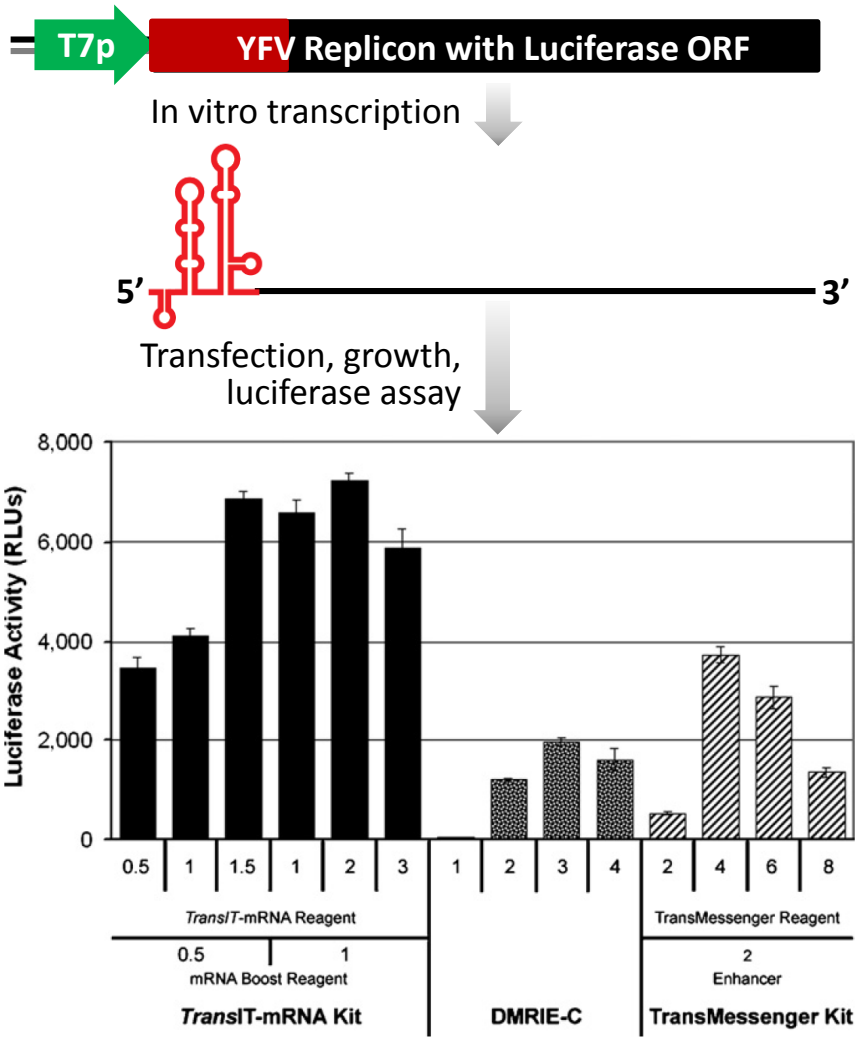
Applications
- Protein:protein interactions
- Structure studies
- Enzyme/functional assays

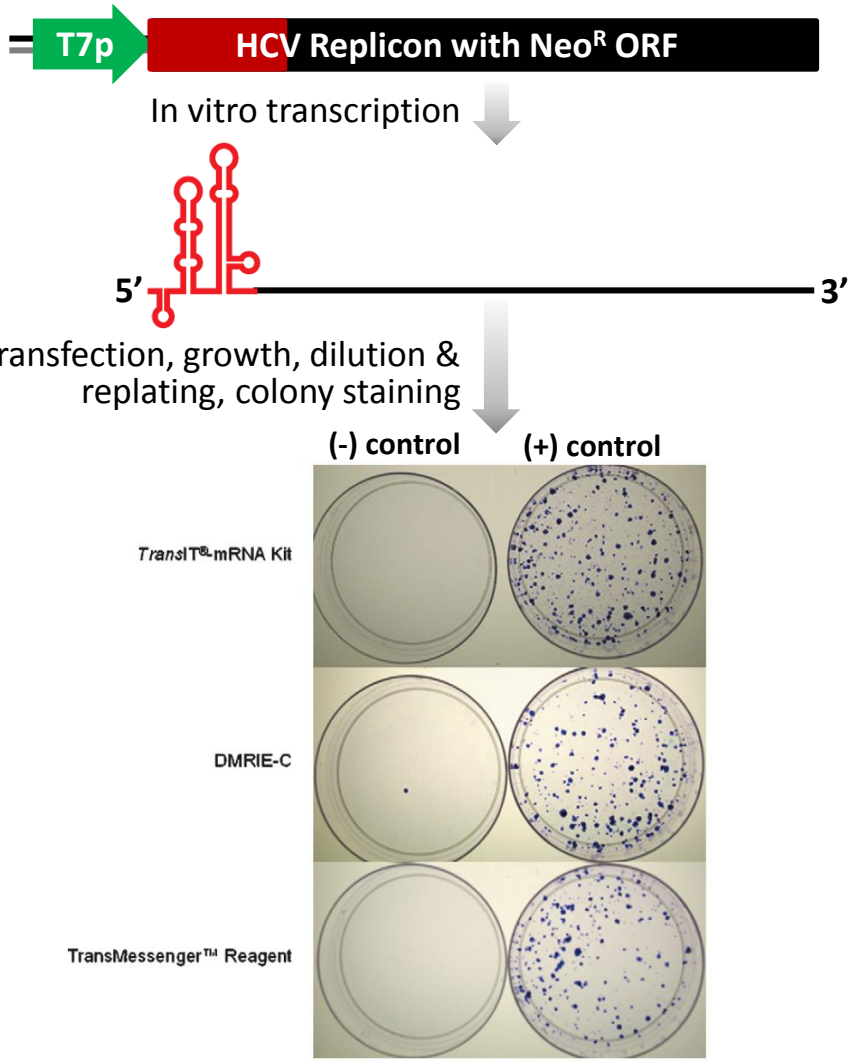
Benefits
- Transient/short term expression
- Pulse of expression
- No potential DNA effects

Applications
- RNA processing, stability studies
- Protein expression
  - e.g. CRISPR nucleases

Transfection of YFV, HCV Viral RNAs Made In Vitro

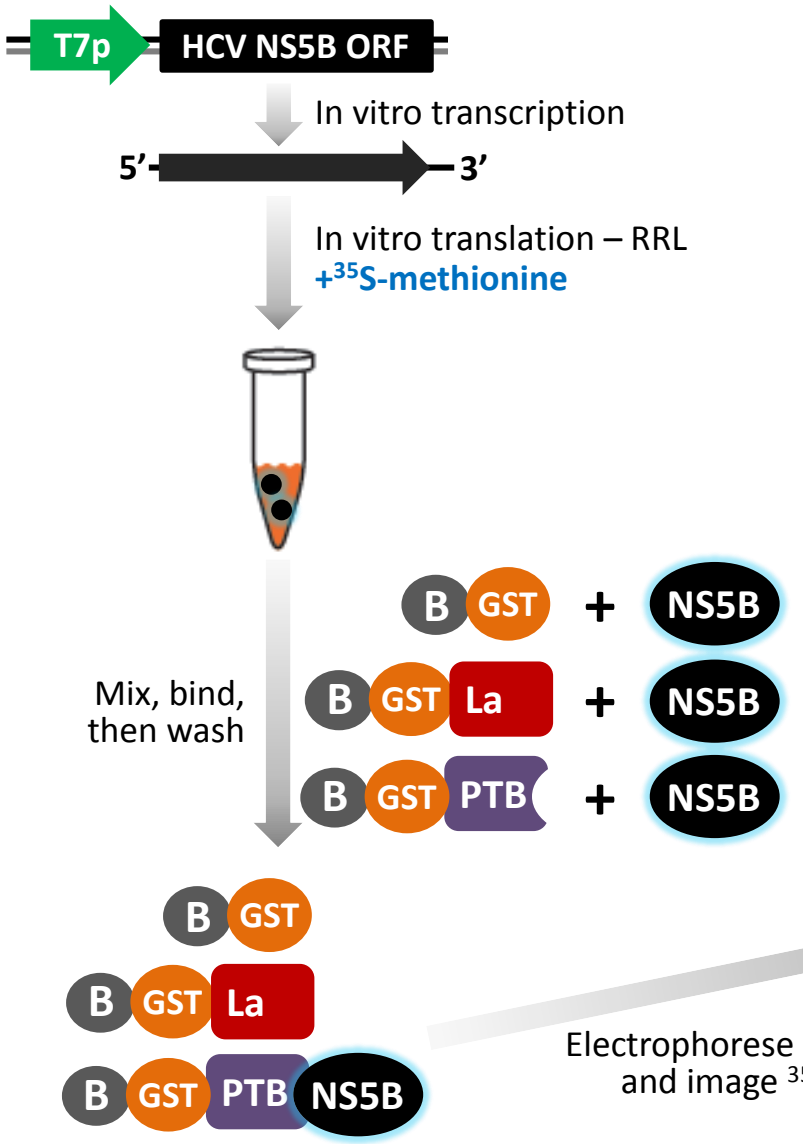
Transfected RNAs are Translated and Initiate Replication



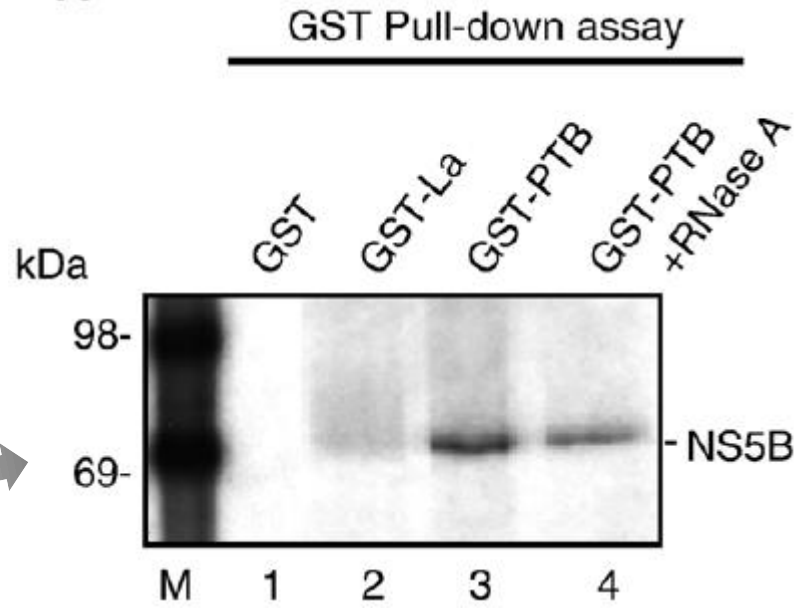


From: Gonzalez et. al. (2007) Selection of an optimal RNA transfection reagent and comparison to electroporation for the delivery of viral RNA. *Journal of Virological Methods.* **145**:14-21

Using In Vitro Transcription and Translation to Identify HCV Protein: Host Protein Interactions by GST Pull-downs



From: Domitrovic et. al. (2005) Role of La autoantigen and polypyrimidine tract-binding protein in HCV replication. *Virology.* **335**:72-86



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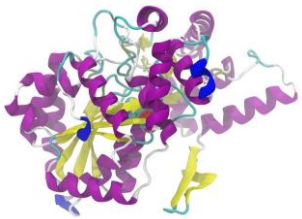
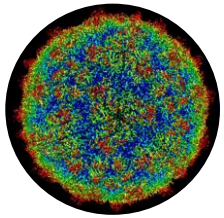
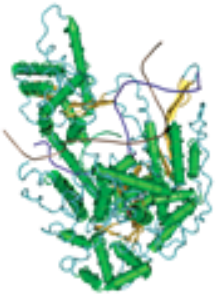
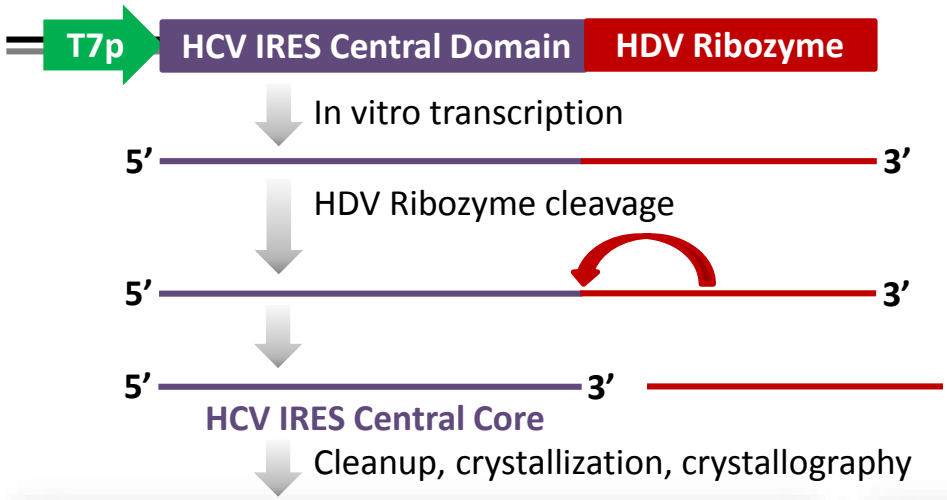




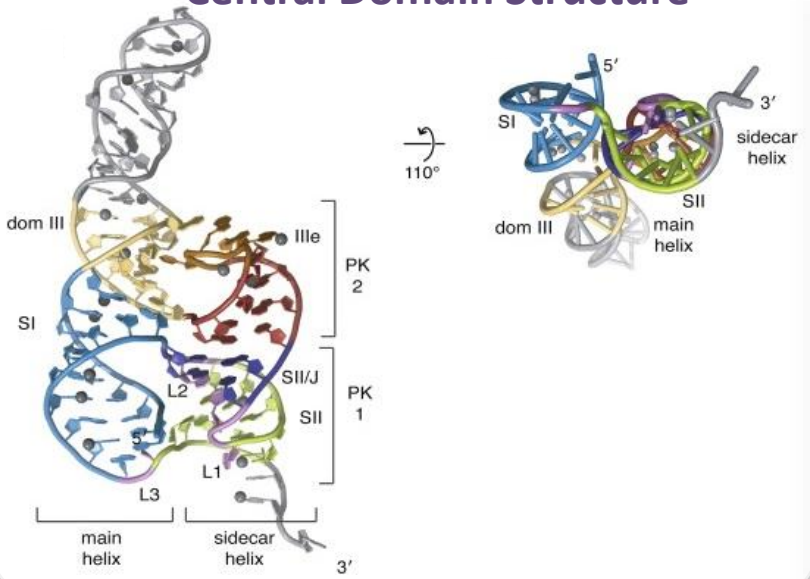


Using In Vitro Transcribed HCV IRES RNA to Determine Its Crystal Structure

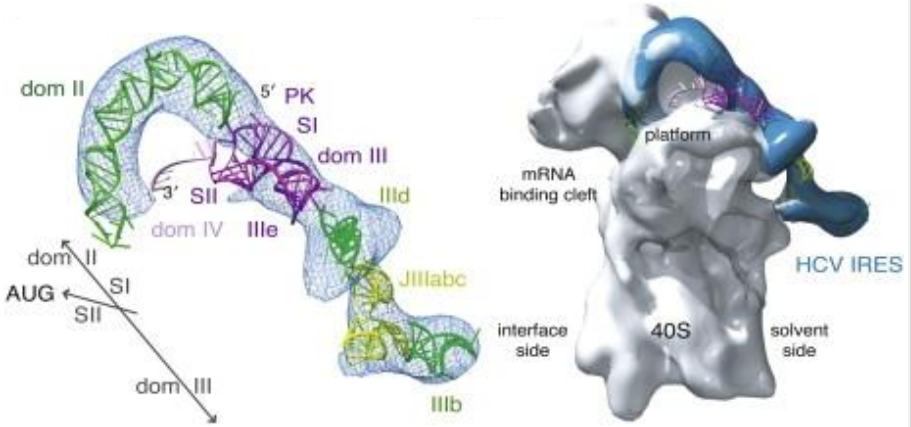


From: Berry et. al. (2011) Crystal Structure of the HCV IRES Central Domain Reveals Strategy for Start-Codon Positioning. *Cell.* **19**:1456-1466

Central Domain Structure



Model of IRES on 40S Ribosomal Subunit



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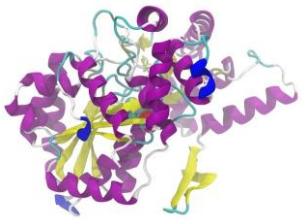
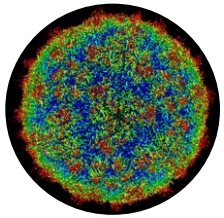
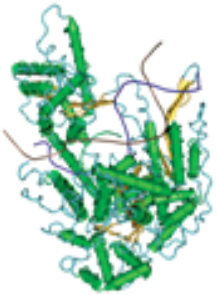






What Are RNA Aptamers?

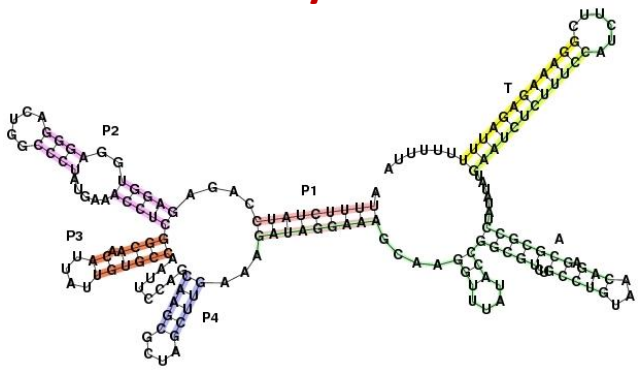
Think of Them as RNA-based Antibodies

Single-stranded RNA molecules that:

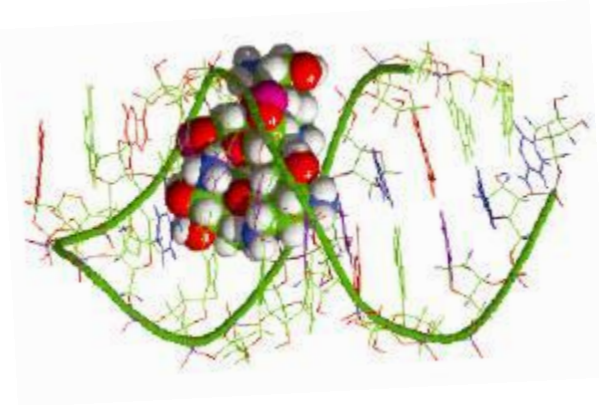
- Fold into RNA structure with secondary and tertiary structure
- Generally 40 to 100 nucleotides long
- Correct sequences/structures bind tightly and specifically to a target molecule (e.g., protein, toxin, cell receptor, other RNAs…)

Think "Antibody made of RNA"…

ssRNA Aptamer with Predicted Secondary Structure

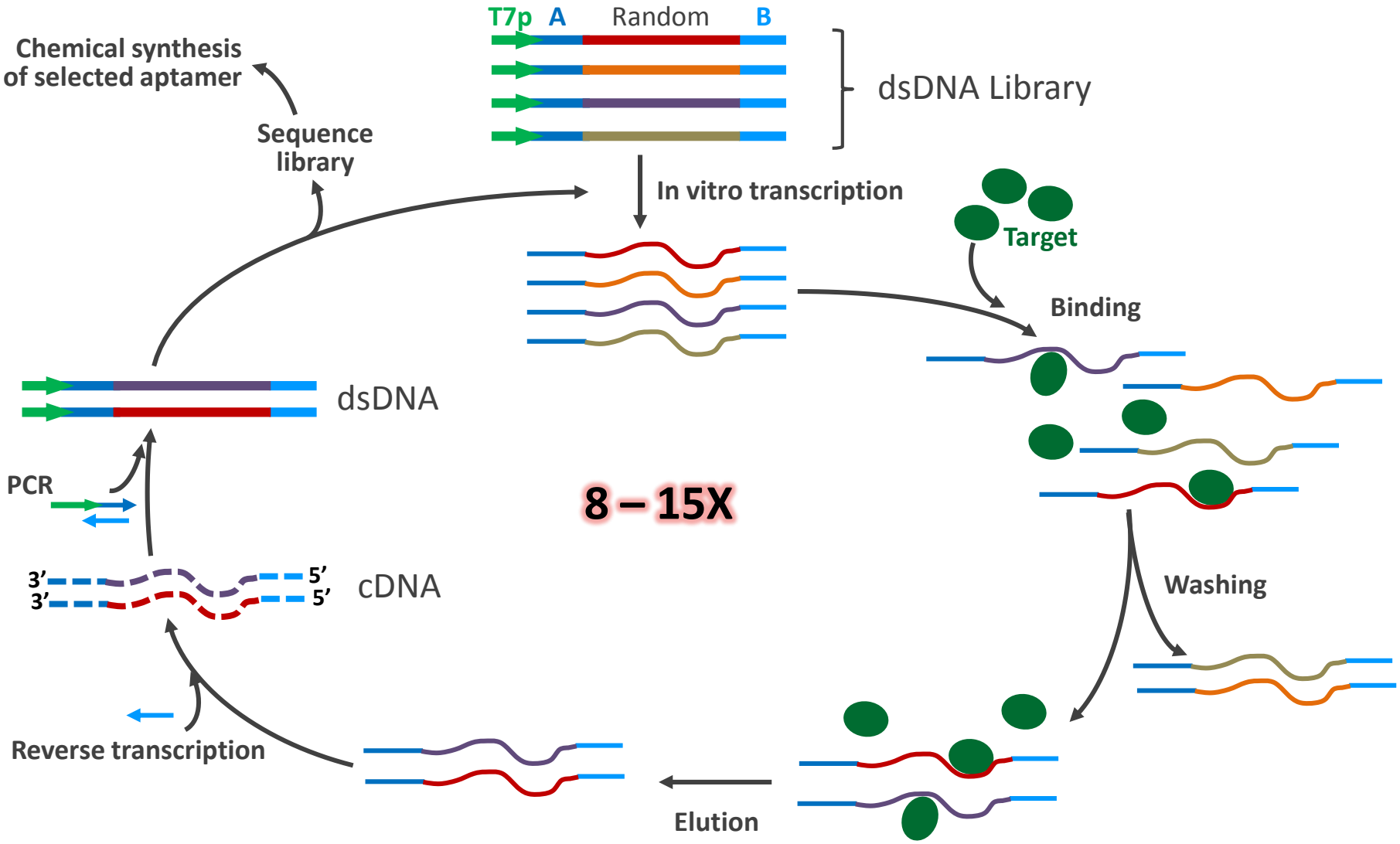


Folds into 3D structure that tightly and specifically binds its target



How Are Good RNA Aptamers Made and Identified? SELEX

Systematic Evolution of Ligands by EXponential Enrichment



Identification of Aptamers that Bind HCV NS5B Replicase

Used Both Standard RNA and 2'-F-CTP, -UTP Substituted RNA

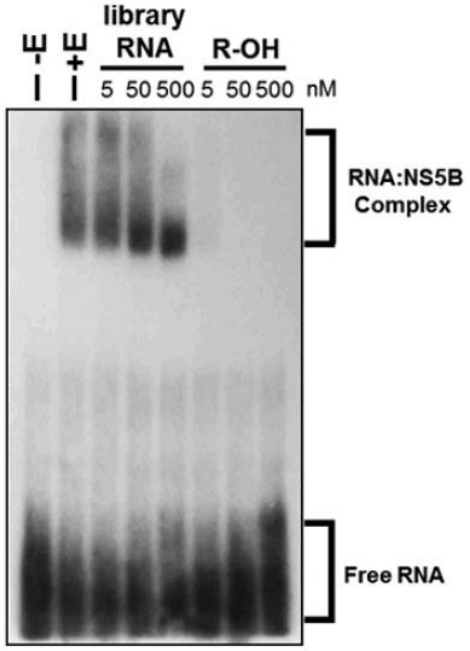
Standard RNA Aptamers Identified

	Sequence	(Occurrence)		Kd (nM)	%
	#1	(6)	5'-UCGAUAAA**AGGGG**CCUGGGAUUGA**AUCGCAU**G**GCCGUG**UC-3'	1.0 ± 0.4	43.9
R-OH	#2	(2)	5'-ACAUUGUG**AGGGG**CUCAGGUGG**AUCGCAU**G**GCCGUG**UC-3'	1.4 ± 1.0	73.5
	#3	(2)	5'-UCGGCU**AGGGG**GUCUGGGCGA**AUCGCAU**U**GCCGUG**CAUC-3'	1.6 ± 0.4	42.2
	#4	(3)	5'-UAAGAGGCUGCAGACCCUUGUGUUAUACUUGAGGAUUUCG-3'	3.0 ± 1.2	65.9

Experimental Design

- Used synthetic DNA library with T7 promoter to synthesize RNA aptamer library
- Used immobilized NS5B replicase as target
- Identified (4) standard RNA aptamers
- Demonstrated inhibition of HCV replication

Aptamer:NS5B Gel Shift Binding Assay



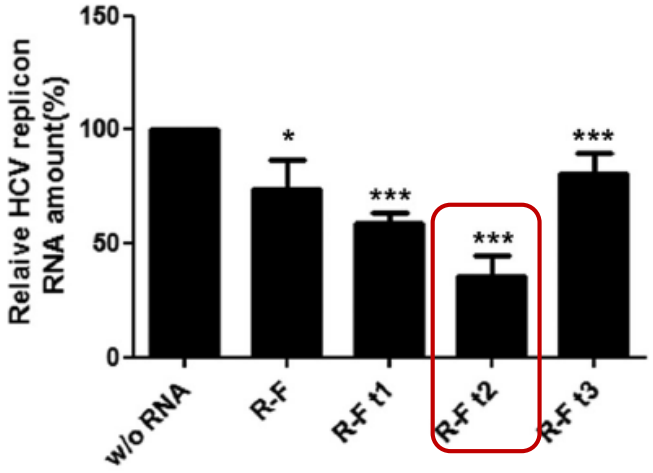
From: Lee et. al. (2013) Inhibition of Hepatitis C Virus (HCV) Replication by Specific RNA Aptamers against HCV NS5B RNA Replicase. *J. Virol.* **87**:7064–7074

Identification of Aptamers that Bind HCV NS5B Replicase
Used Both Standard RNA and 2'-F-CTP, -UTP Substituted RNA

Experimental Design

- Same experimental design but used DuraScribe® T7 Transcription Kit to make RNase A-resistant aptamers
- Durascribe® Kits generate 2'-F-UTP, -CTP substituted RNAs (aptamers here)
- Identified one high affinity 2'-F-Aptamer, R-F
- Also inhibited HCV replication

Inhibition of an HCV Replicon



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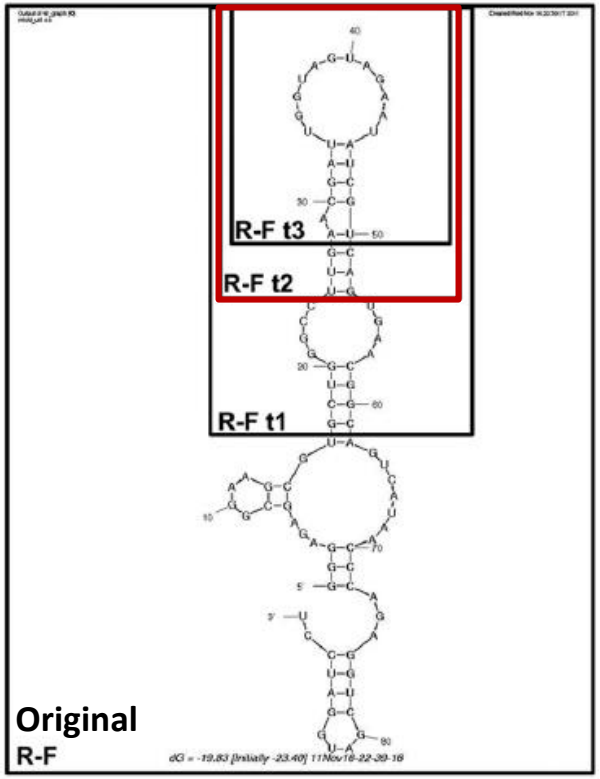
Full-Length & Truncated R-F Aptamers



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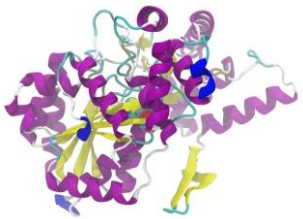
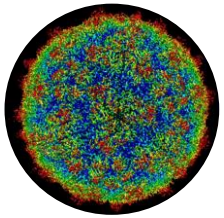
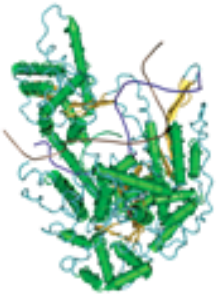




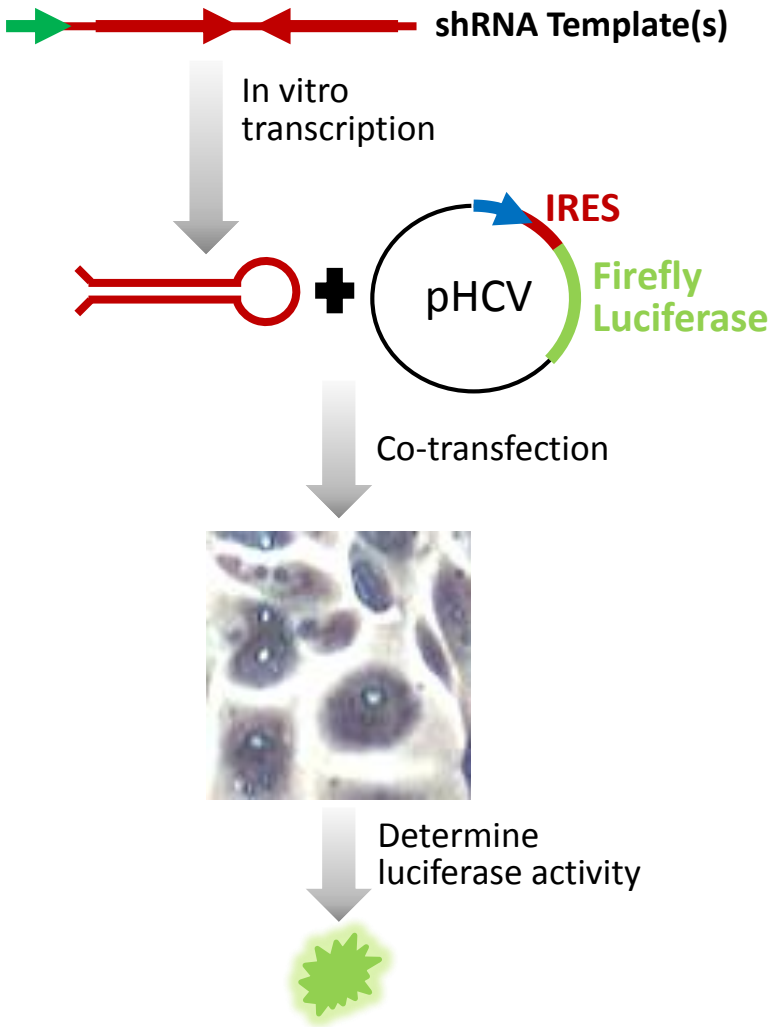


RNA Interference using shRNAs Targeting HCV

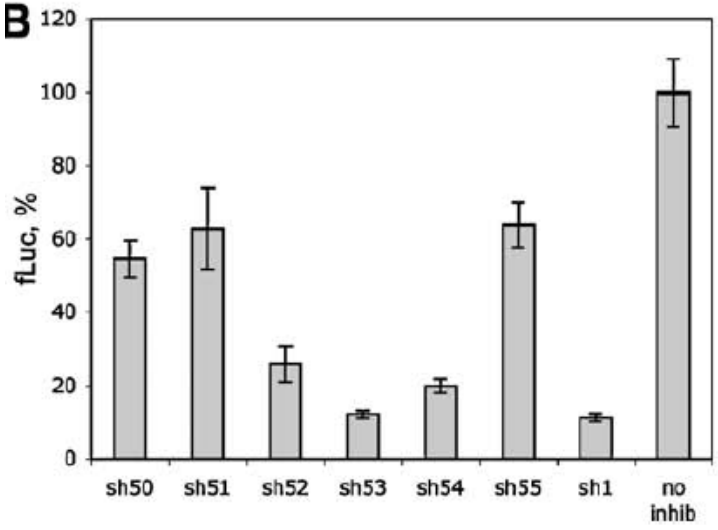
IVT shRNAs Induced RNAi and mRNA Cleavage

Experimental Design

- Designed shRNAs targeting the HCV IRES
- Transcribed those shRNAs using the AmpliScribe™ T7-FLASH Transcription Kit
- Cotransfected shRNA into cells transfected with HCV IRES-driven luciferase reporter plasmid (+control)



Inhibition of a pHCV Luciferase Expression



From: Vlassov et. al. (2007) shRNAs Targeting Hepatitis C: Effects of Sequence and Structural Features, and Comparison with siRNA. *Oligonucleotides* **17**:223–236

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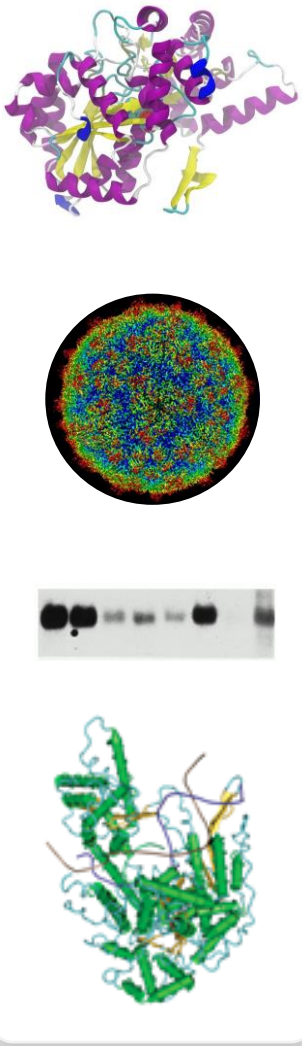
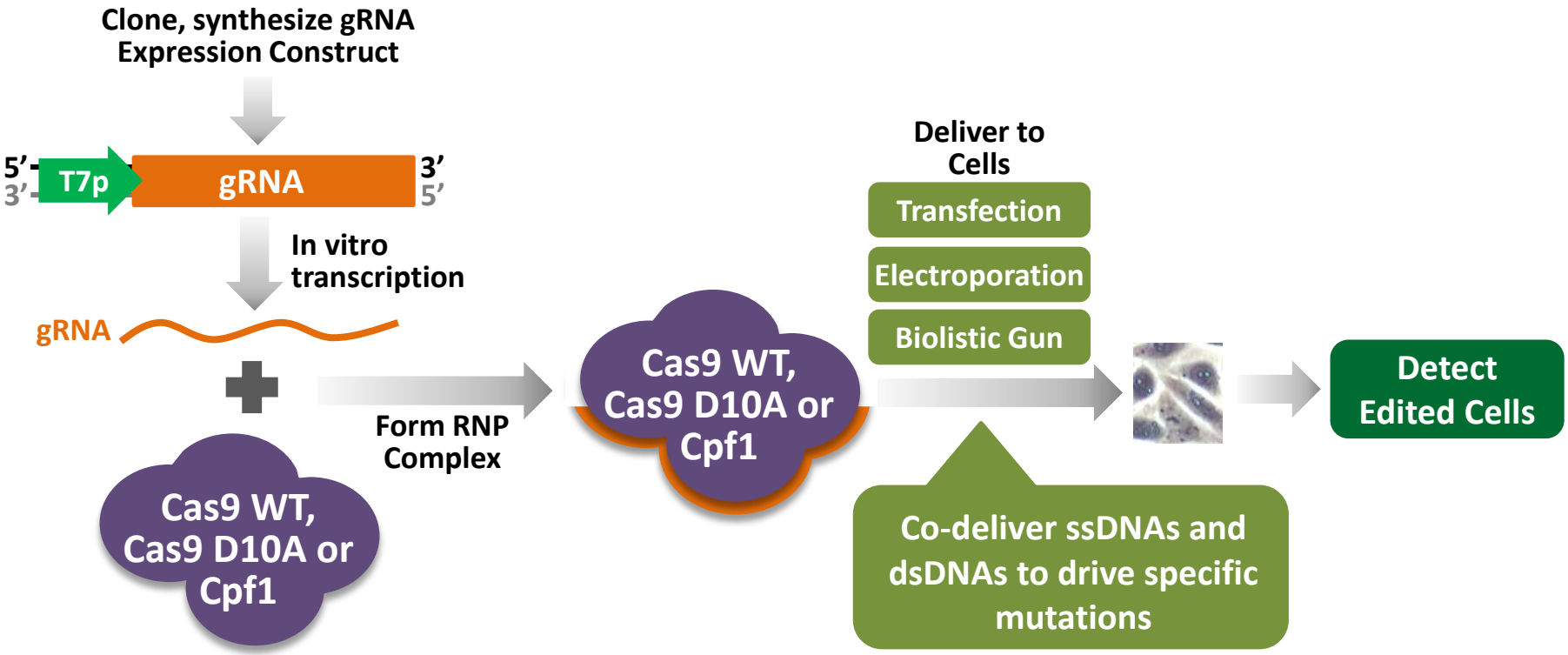


CRISPR Gene Editing by RNP Delivery

Synthesis of Guide RNAs by IVT



CRISPR Gene Editing by RNP Delivery

Synthesis of Guide RNAs by IVT

Experimental Design

- Designed gRNAs targeting 4 different genes in maize
- Transcribed those gRNAs using the AmpliScribe™ T7-FLASH Transcription Kit
- Complexed with Cas9 protein and delivered to maize embryos by biolistic particle transformation
- Amplified target regions and sequenced amplicons to identify mutation frequencies & changes
- Also, co-delivered ssDNA "repair oligo" to change ALS2 proline 165 to a serine and grew up edited plants

Common Mutations

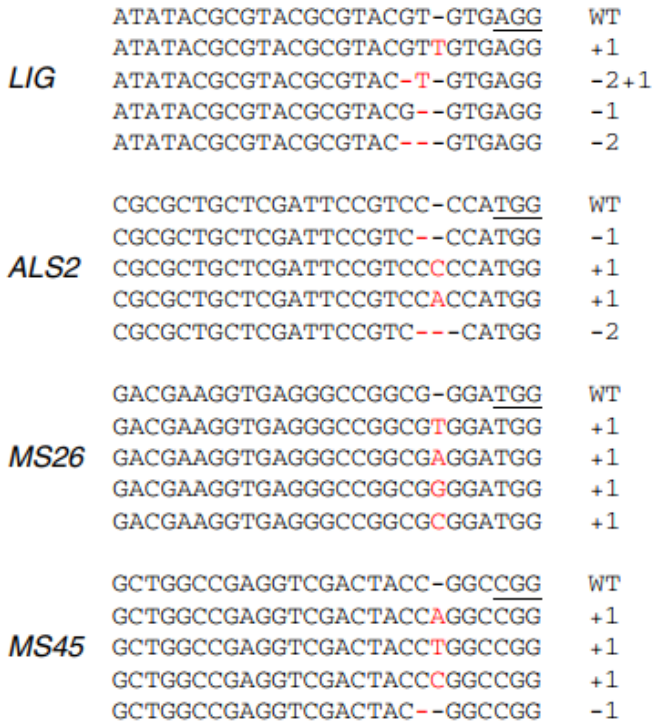


Table 1 | Mutation frequencies at the intended and MS45 off-target sites upon delivery of Cas9 and gRNAs as DNA vectors or RNP complexes into maize immature embryo cells.

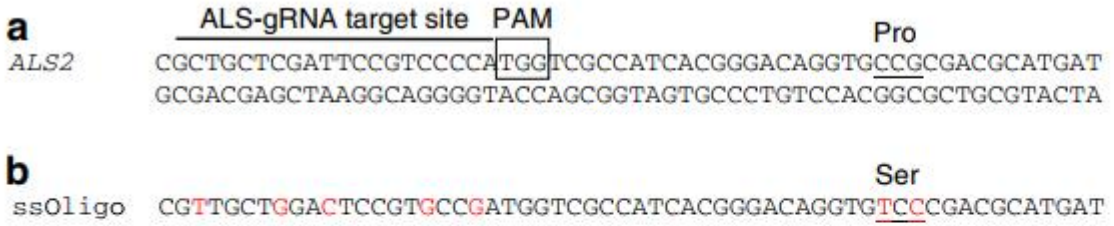
Target site	Target site sequence with PAM*	Cas9 only (%)	DNA delivery (%)†	RNP delivery (%)†
LIG	GCGTACGCGTACGTGTGAGG	0.004	0.56	0.57
ALS2	GCTGCTCGATTCCGTCCCCATGG	0.020‡	0.51	0.45
MS26	GCACGTACGTCACCATCCCGCCGG	0.004	0.43	0.21
MS45	GGCCGAGGTCGACTACCGGCCGG	0.002	0.34	0.69
MS45 off-site	**C**GCCGAGG**G**CGACTACCGGCAGG	0.002	0.18	0.01

From: Svitashev et. al. (2016) Genome editing in maize directed by CRISPR–Cas9 ribonucleoprotein complexes. *Nat. Comm.* **7**:13274

CRISPR Gene Editing by RNP Delivery

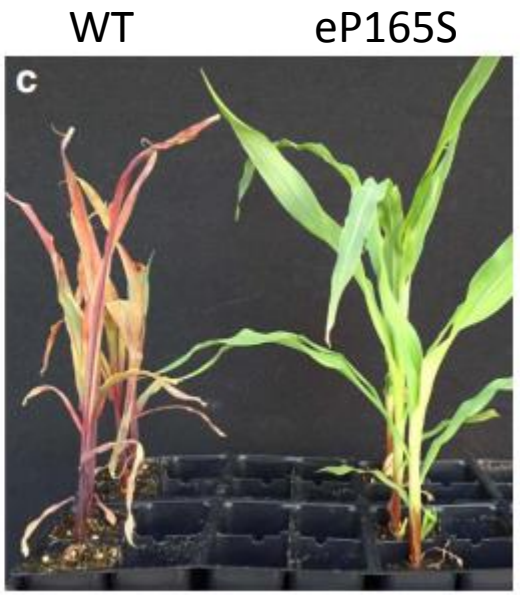
Generating Herbicide Resistant Plants

Guide RNA to ALS Gene and "Helper Repair Oligo"



- Proline to serine mutation confers herbicide resistance
- **Right plant**: Edited plant, herbicide resistant
- **Left plant**: Wild type, herbicide sensitive

Test Plants Treated with Herbicide



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Uses/Applications of IVT RNA

IVT RNA Makes Many Experiments Possible

- ✓ Viral RNA synthesis and transfection to start replication
- ✓ mRNA synthesis for in vitro and in vivo applications
- ✓ RNA structure studies
- ✓ RNA aptamer synthesis for SELEX
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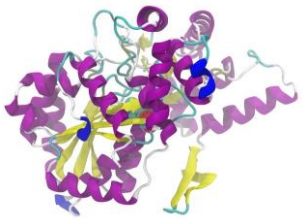
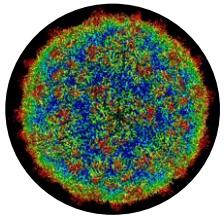
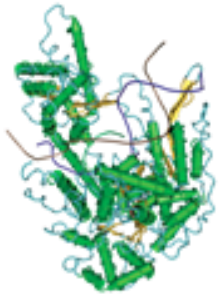


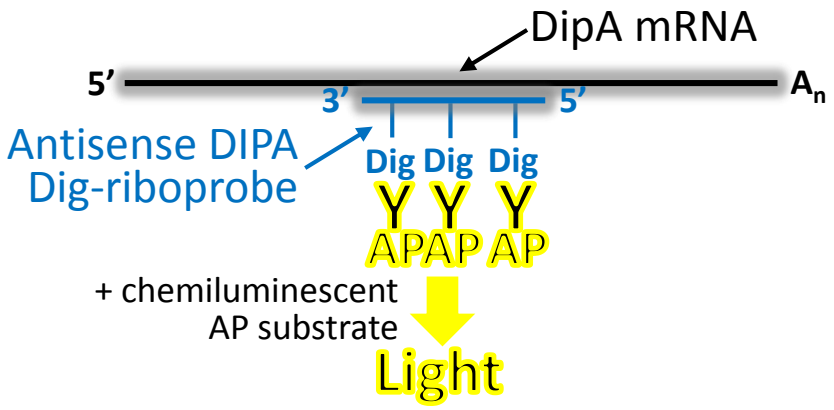




Northern Blotting with a Dig-labeled Riboprobe

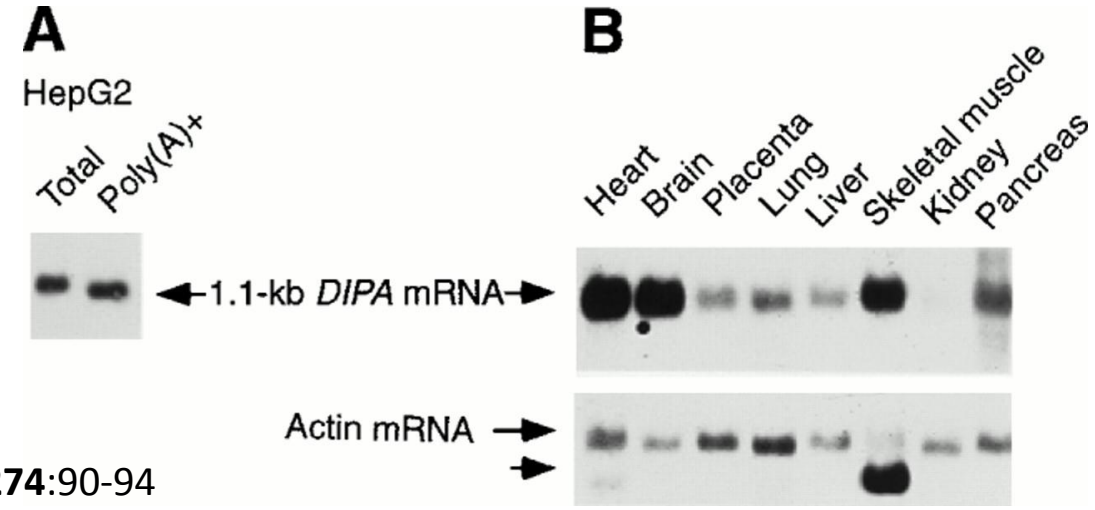
Determining Expression Levels of a New Cellular Transcript

Experimental Design



- Ran total RNA on formaldehyde gel
- Transferred RNA to a membrane
- Hybridized antisense Dig-DIPA riboprobe
- Washed and probed with alkaline phosphatase conjugated anti-dig antibody
- Washed and detected bound antibody (DIPA mRNA) with chemiluminescent substrate and film!

Northern Blot Results



Brazas and Ganem (1996) *Science* **274**:90-94

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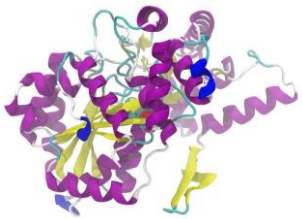
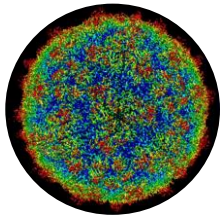
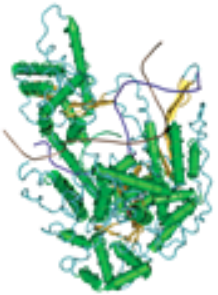


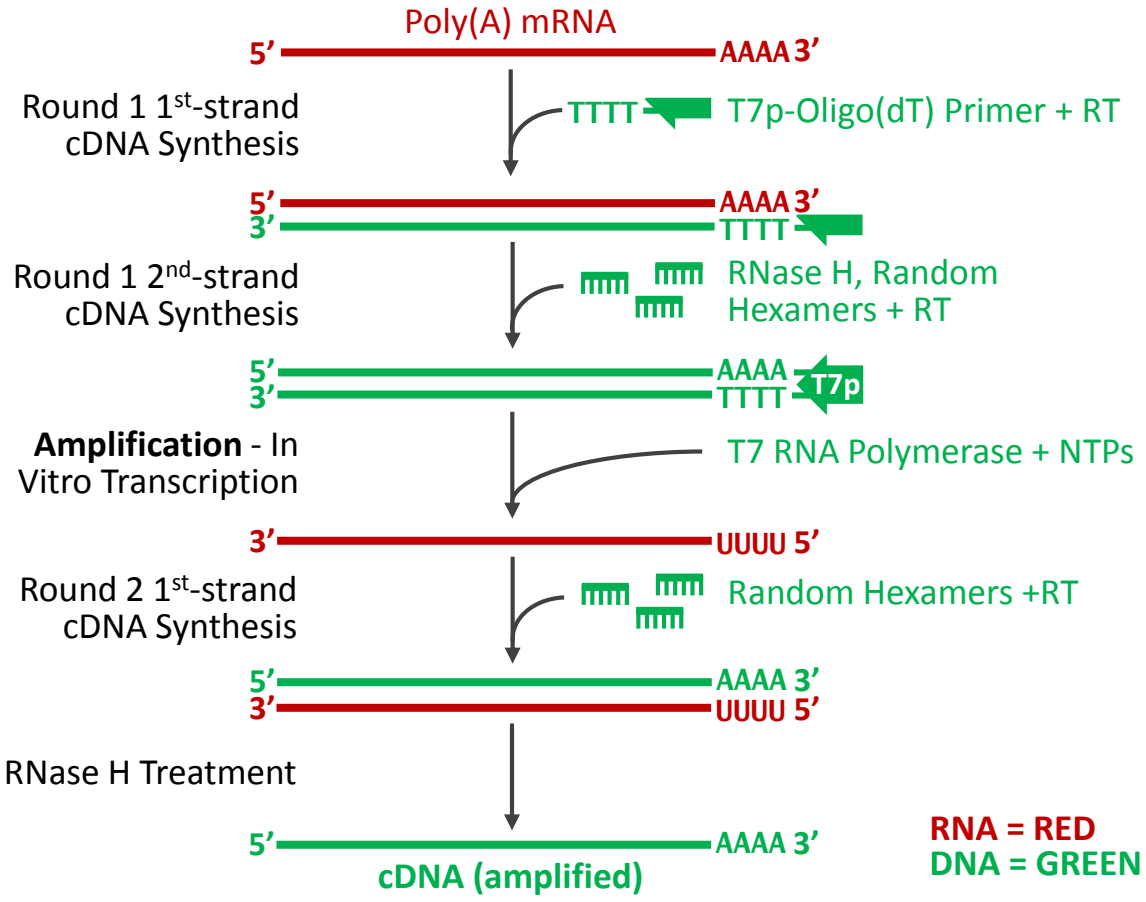




Using IVT to Amplify RNA in Limiting Samples

Produce Amplified cDNA Without Altering mRNA Profiles

MessageBOOSTER™ qPCR Kit Protocol



- Linear RNA amplification process preserves transcripts relative abundance
- Perform more RT-qPCR reactions from precious samples
- Readily and reproducibly detect even low-abundance transcripts in RNA from a single cell
- Produce enough cDNA to archive for later use

Key Challenges Associated with In Vitro Transcription

Time and Yield Are the Main Challenges

- **Time**
  - Many IVT reactions take 2-3 hours to produce maximal RNA yields
- **Yield**
  - Low yield kits/protocols mean more repeat IVT reactions
  - High yield kits decrease the number of reactions needed to produce enough RNA – decreasing costs and time spent
- **RNA degradation**
  - RNA is very sensitive to degradation by RNases!
  - Some of those applications require multiple steps and present many opportunities for RNases to degrade your precious RNA (e.g SELEX)
- **Synthesizing both long and short RNAs**
  - Transcribing full-length long RNAs can be difficult due to secondary structure, GC content… (e.g. critical for RNA virus genome studies)
  - Short RNAs can also be difficult to produce due to minimal length of templates

AmpliScribe™ T7-FLASH Transcription Kit

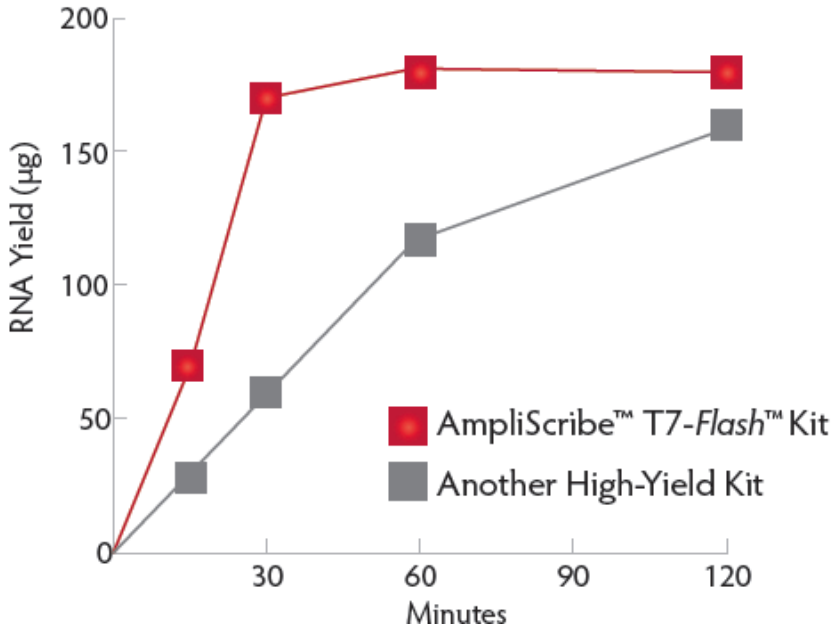
Solves the Time and Yield Challenges of IVT

- **Fast**
  - Produce maximal RNA yields in only 30 minutes.
- **High Yields**
  - Produce 180 µg of RNA from only 1 µg of template DNA.
- **Flexible**
  - Accepts a variety of template DNAs with T7 promoters including linearized vectors, PCR products, cDNA, and dsDNA oligos.
- **Scalable**
  - Reactions can be scaled up to quickly produce milligram - gram amounts of RNA.
- **Multi-application compatible**
  - Make long (9 kb in house, >11 kb by researchers)
  - Make short (>25 bases) RNA transcripts
  - Suitable for all the applications discussed today and more

AmpliScribe™ T7-FLASH Transcription Kit

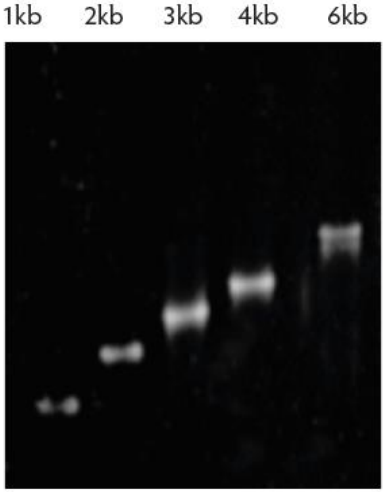
Higher Yield than Competitor Kits in Only 30 Minutes

Higher Yields in 30 Minutes vs 60 Minutes with Competitor Kits



- 1.4 kb template
- 1 µg of template DNA/rxn

High Yields from Both Small and Large Templates



Transcript Size	Yield (µg)
1 kb	192
2 kb	185
3 kb	191
4 kb	206
6 kb	187

- Various template sizes
- 1 µg of template DNA/rxn
- 30 minute reactions

AmpliScribe™ T7-FLASH Transcription Kit

High Yields of Small RNA or from Limiting Template Input

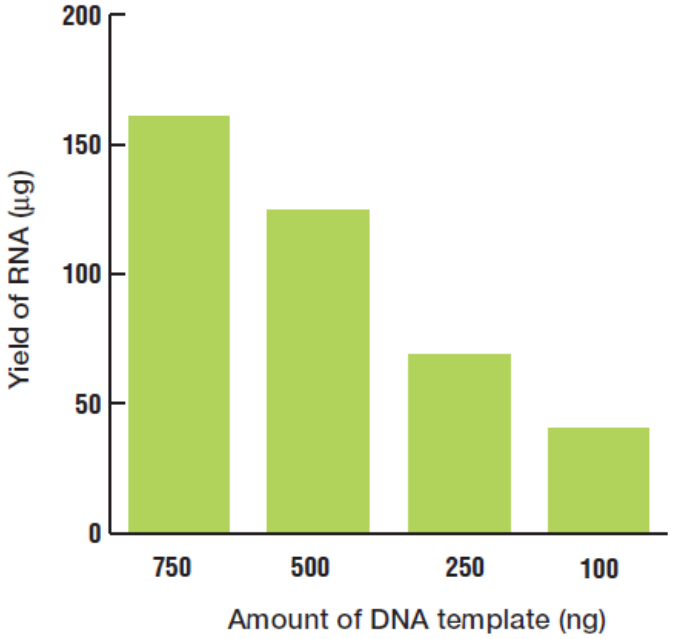
High Yields of Very Short Transcripts (>26 bases)

RNA Transcript Size	RNA Yield (µg)	RNA Yield (pmoles)
26 bases	12	1319
47 bases	24	1459
96 bases	36	1071
335 bases	76	648
1.4 kb	176	359
6 kb	187	89

- Various template sizes
- 1 µg of template DNA/rxn
- 30 minute reactions

Note: Increasing rxn temp, time, & template amounts increases short transcript yields

Yields Remain High With Decreasing Template Amounts



- Various template amounts
- 1.4 kb transcript
- 30 minute reactions

DuraScribe® T7 Transcription Kit

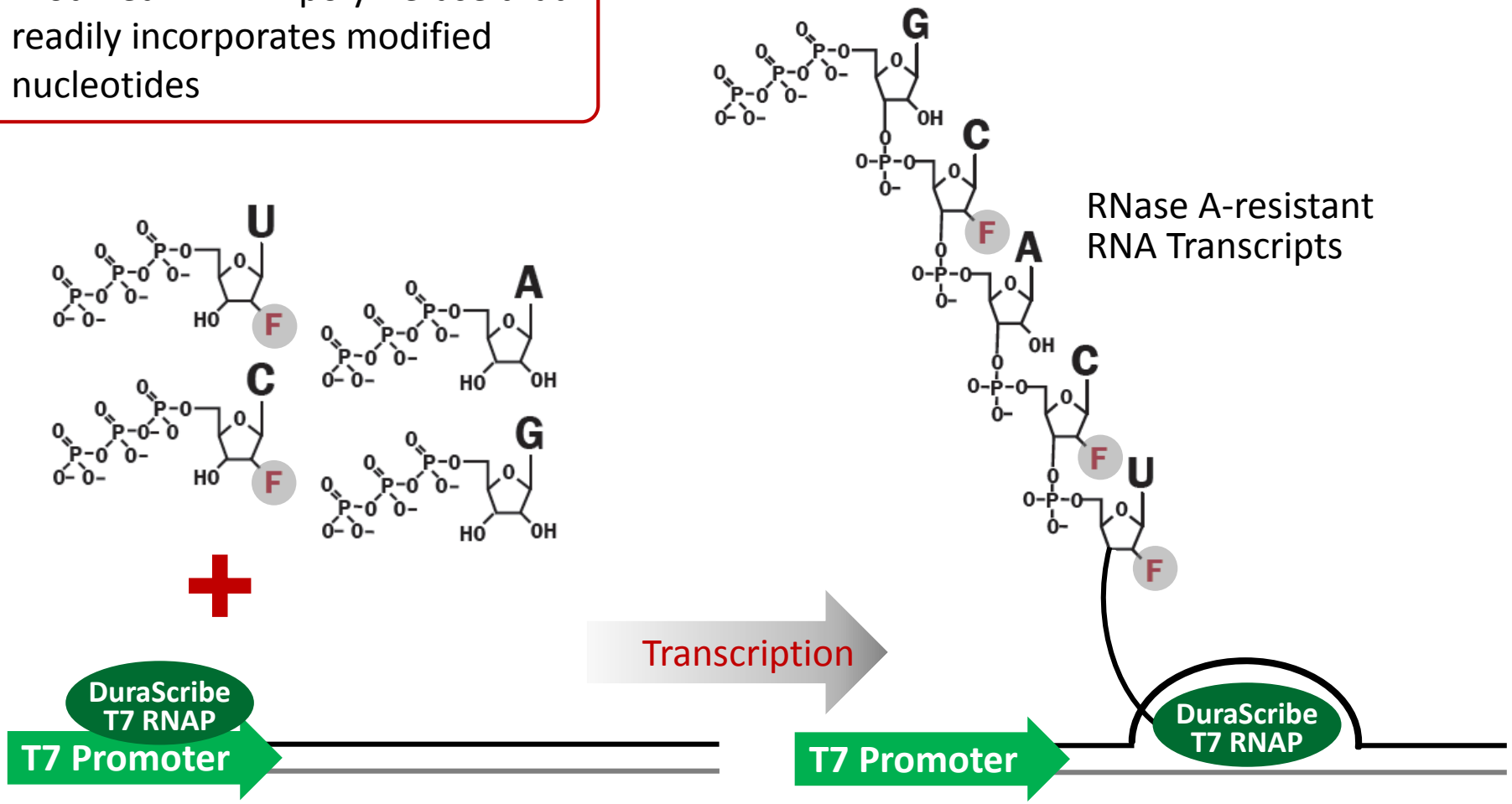
RNase A-resistant Transcripts for Demanding Applications

- **Stable**
  - DuraScript® RNA transcripts are fully resistant to RNase A
  - Protects RNA during complicated experiments like SELEX or in harsh environments like tissue culture media
- **High Yields**
  - Produce ~40 - 60 µg of DuraScript RNA from only 1 µg of a 1.4 kb template in 4 – 6 hours
  - 110 – 307 pmol of transcripts depending on the length
- **Flexible**
  - Uses the same T7 promoters and templates as standard T7 RNA polymerase (e.g. AmpliScribe™ Kits)
- **Multi-application compatible**
  - Make long or short transcripts
  - Creates suitable substrates for RNAi, (dsRNA for dicing), antisense RNA, miRNA and aptamer identification
  - Not suitable for experiments requiring translation

How Does the DuraScribe® T7 Transcription Kit Work

Incorporates 2’-F-CTP & 2’-F-UTP with a Modified T7 RNAP

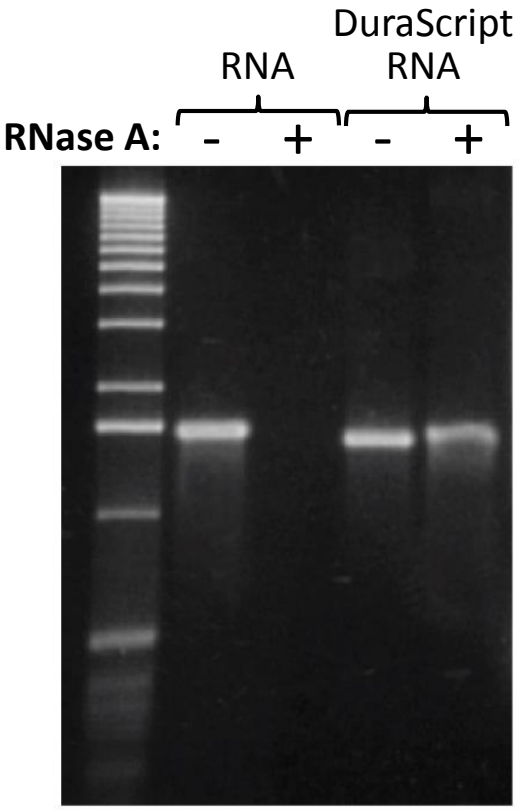
DuraScribe® T7 RNA Polymerase: Modified T7 RNA polymerase that readily incorporates modified nucleotides



DuraScribe® T7 Transcription Kit

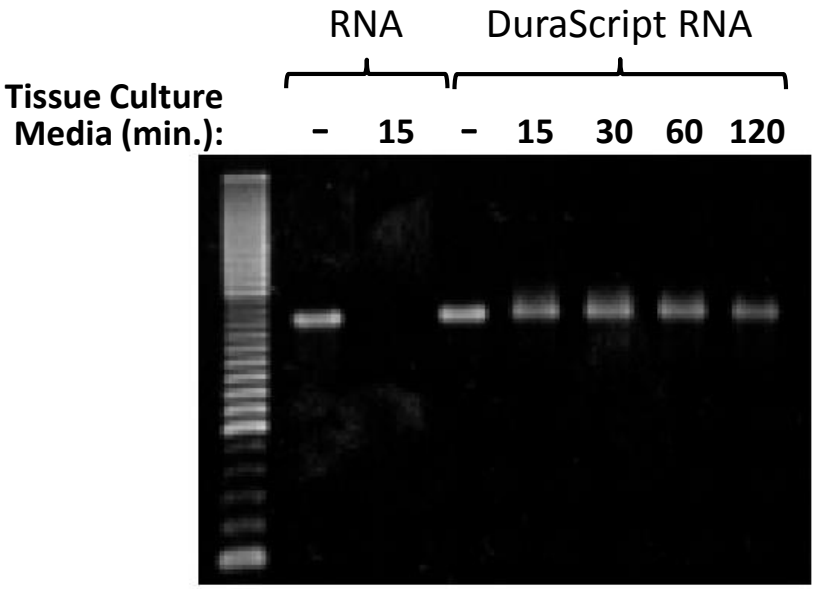
Produce Stable, RNase A-resistant Transcripts

Resistant to RNase A



- 1.4 kb transcripts (standard, DuraScript®)
- +/- 1 unit high purity RNase A
- 30 minute incubation, then gel analysis

DuraScript RNA is Stable in Tissue Culture Media for ≥2 Hours

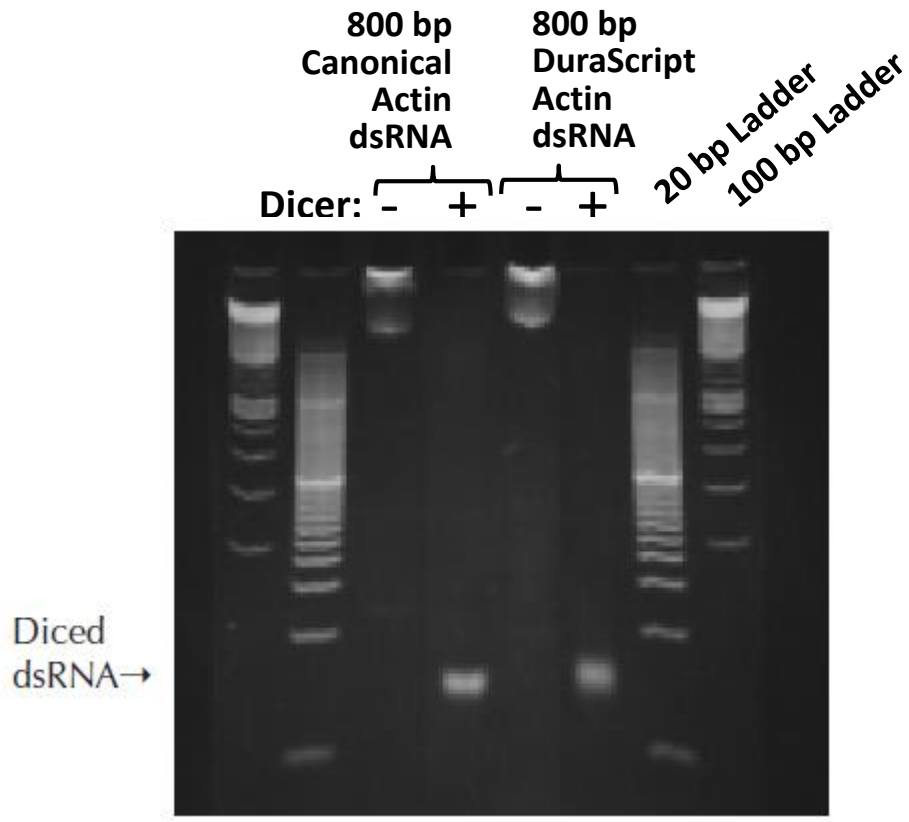


- 1.4 kb transcripts (standard, DuraScript®)
- +/- 100 µL D-MEM + 10% FCS @37°C
- Incubated for indicated times and then analyzed by gel electrophoresis

DuraScribe® T7 Transcription Kit

DuraScript® dsRNA are Effective RNAi Substrates

DuraScript dsRNA is Digested by Recombinant Human Dicer Enzyme as Efficiently as Canonical dsRNA



- 800 bp dsRNA transcripts (standard, DuraScript®)
- 30 µg each RNA digested overnight at 37°C with 30 U of recombinant human dicer enzyme
- Diced RNAs were purified by and analyzed by non-denaturing PAGE

DuraScribe® T7 Transcription Kit

DuraScript® dsRNA are Effective RNAi Substrates

High Yields of RNase A-resistant DuraScript RNA

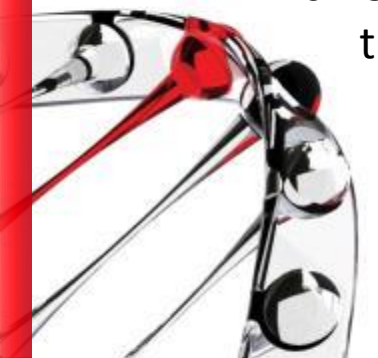
Size of DuraScript RNA Produced	DuraScript RNA Yield	
	(µg)	(pmol)
2600 nts	100 µg	116 pmol
1400 nts	58 µg	124 pmol
330 nts	18 µg	164 pmol
88 nts	9 µg	307 pmol

- Each reaction used 1 µg of template (templates = same construct digested at 4 locations to produce different length transcripts)
- Reactions were incubated at 37°C for 4 hr and quantified

Summary

IVT Offers a Rapid, Cost-Effective Method of Making RNA for a Wide Variety of Applications

- IVT RNA can be used for multiple applications from in vitro or in vivo protein expression through RNAi knockdowns and CRISPR gene editing.
- Key challenges include long IVT reaction times, low yields and RNA degradation
- The AmpliScribe™ Kit overcomes the first two challenges with the fastest reactions (30 min) and the highest yields
- The DuraScribe® Kit offers the unique ability to produce RNase-A resistant RNA
  - Optimal for:
    - Complicated experiments such as SELEX-based aptamer identification
    - Use in harsh environment where degradation may affect your results (e.g. tissue culture)



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techsupport@lucigen.com
(608) 831-9011
8 am – 5 pm central time

Contact me.
Rob Brazas, Ph.D.
Sr. Product Manager
rbrazas@lucigen.com

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