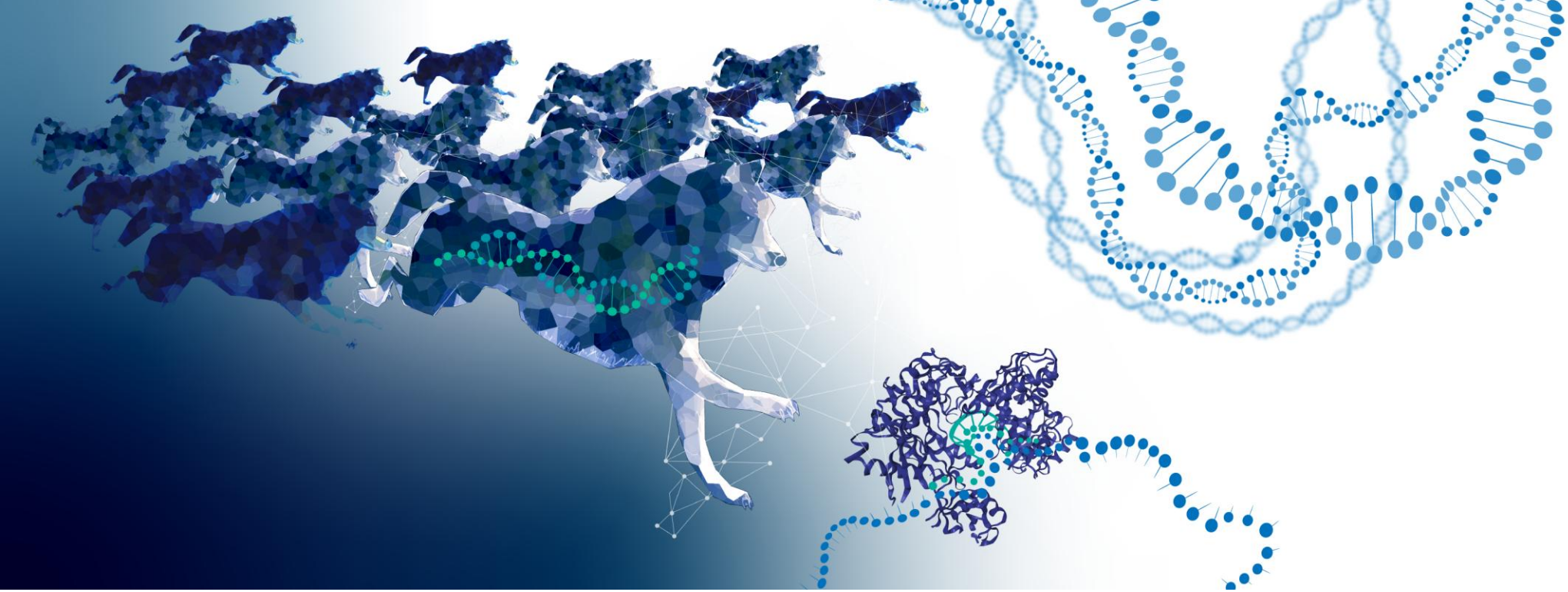


siTOOLs Biotech

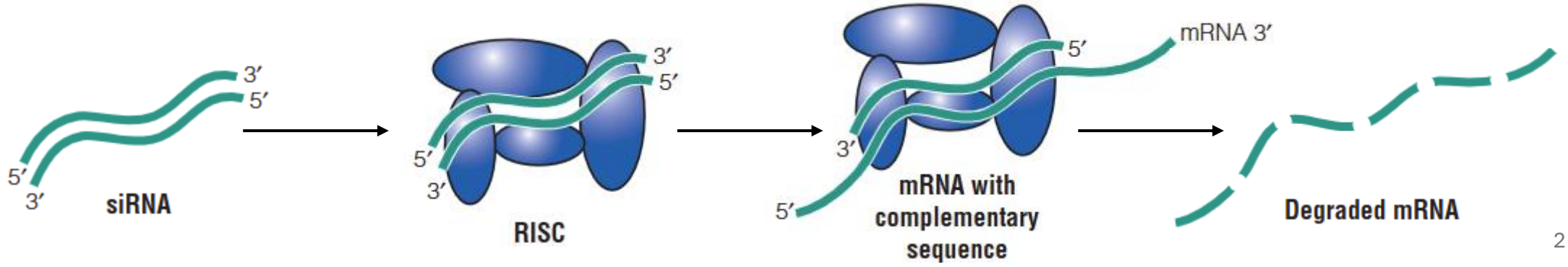
From RNAi to RNAseq

EASTPORT LIFESCIENCE

siTOOLs BIOTECH

Basics of RNA interference

- RNA interference (RNAi) is a natural process in eukaryotes
- Helps protect from exogeneous RNAs and regulates gene function
- Small interfering RNA (siRNA) and micro RNA (miRNA) play vital role in RNAi by binding to specific mRNAs
- Resulting dsRNA is then degraded by the RNA induced silencing complex (RISC)

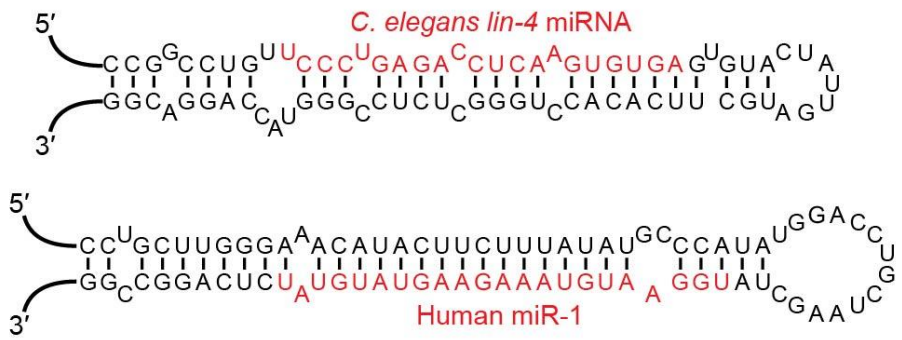


siRNA vs. miRNA

Both miRNA and siRNA have similar function in RNAi but differ in origin and specifity

siRNA

- Derived from exogeneous dsRNA molecules (e.g. viral) or endogeneous hairpin structures
- Double-stranded with size around 21 bp
- Perfect complementarity to the target sequence

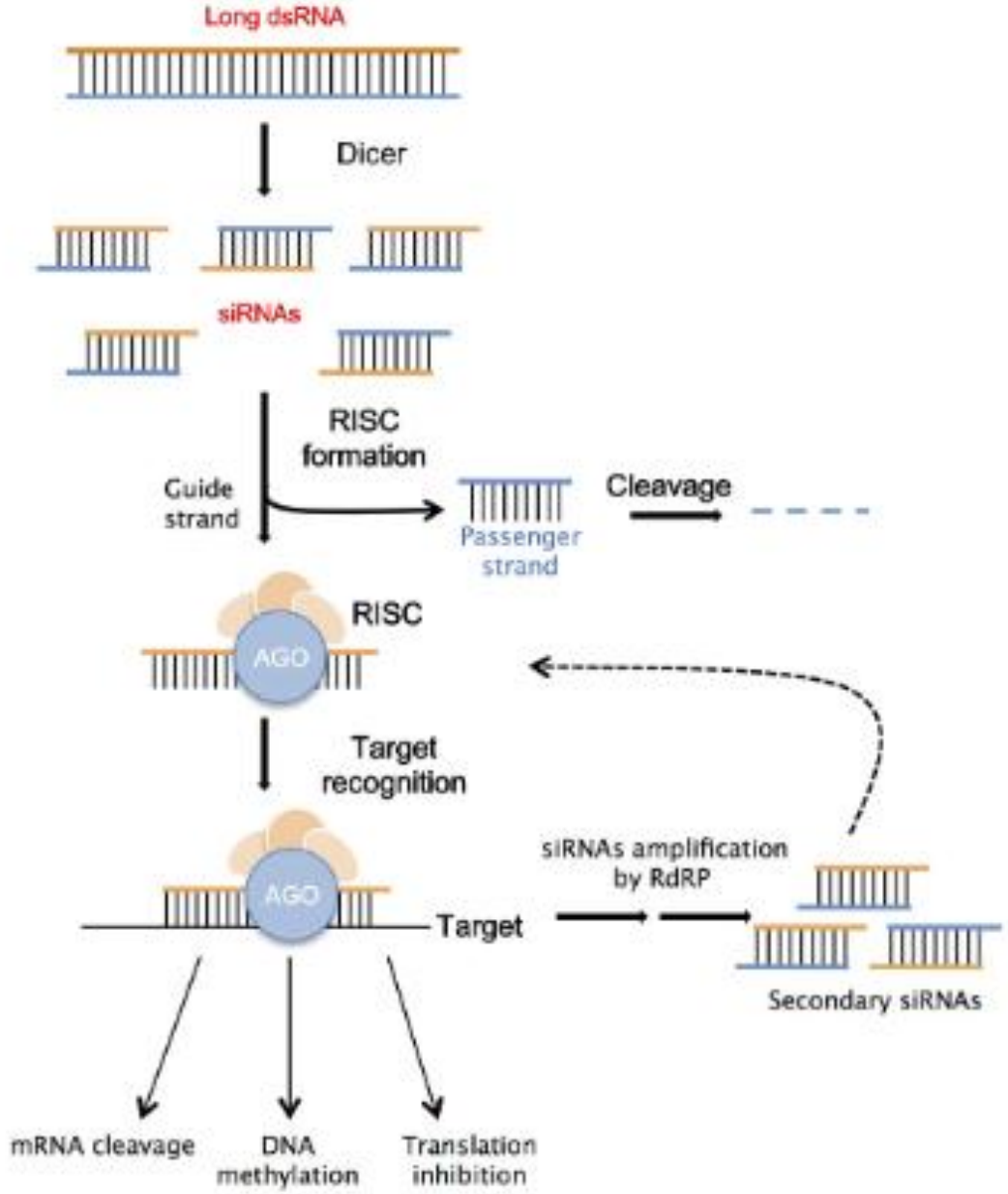


miRNA

- Produced from endogeneous genes in the nucleus
- transcribed as primary miRNA transcripts (pri-miRNA) → processing to mature miRNAs
- Single stranded RNAs that form secondary structures
- Only partial complementarity (6-8 bases) – „seed“ sequence

RNAi Pathway

- The longer siRNA is firstly cleaved by the Dicer endoribonuclease to 21-23 fragments
- siRNA binds to the Argonaute protein in the RISC complex and is unwound
- The RISC complex guides the siRNA or miRNA to the target mRNA molecule
- The complementary mRNA is then cleaved by the complex and its translation into protein is prevented

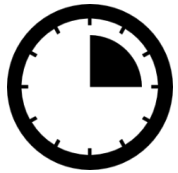


Adapted from Limera et al. (2017) *Front. Plant Sci*. 8:1418.

RNAi Applications

- RNAi can be used for studying gene function by selectively silencing gene of interested
- Therapeutic potential – tailoring to target viral genes, disease genes and certain types of cancer
  - In 2018 Patisiran approved as first siRNA-based treatment for polyneuropathy in hereditary transthyretin-mediated amyloidosis
- Crop research - disease resistance, pest control and increased nutrition
- Functional genomics – studying the function of genes and the effect of knockdown on phenotype

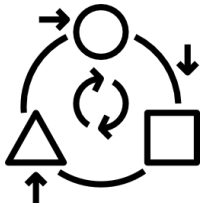
RNAi Benefits



Fast (results in days, not weeks)

Dose-dependent (drug-like)

Simple (no need for engineered cell lines)

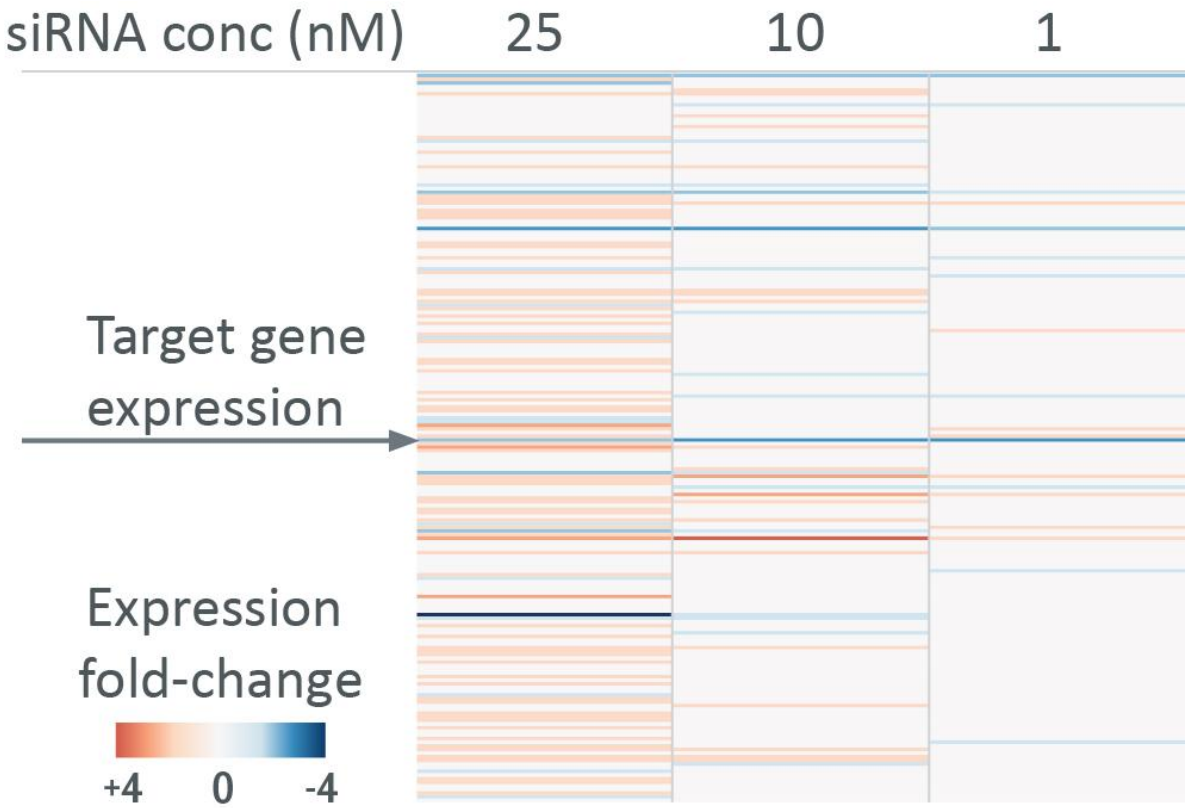


Transient (avoids adaptation)

Broadly applicable in various biological context

RNAi drawback – off target effects

siRNA-induced changes in gene expression after the use single siRNA against STAT3

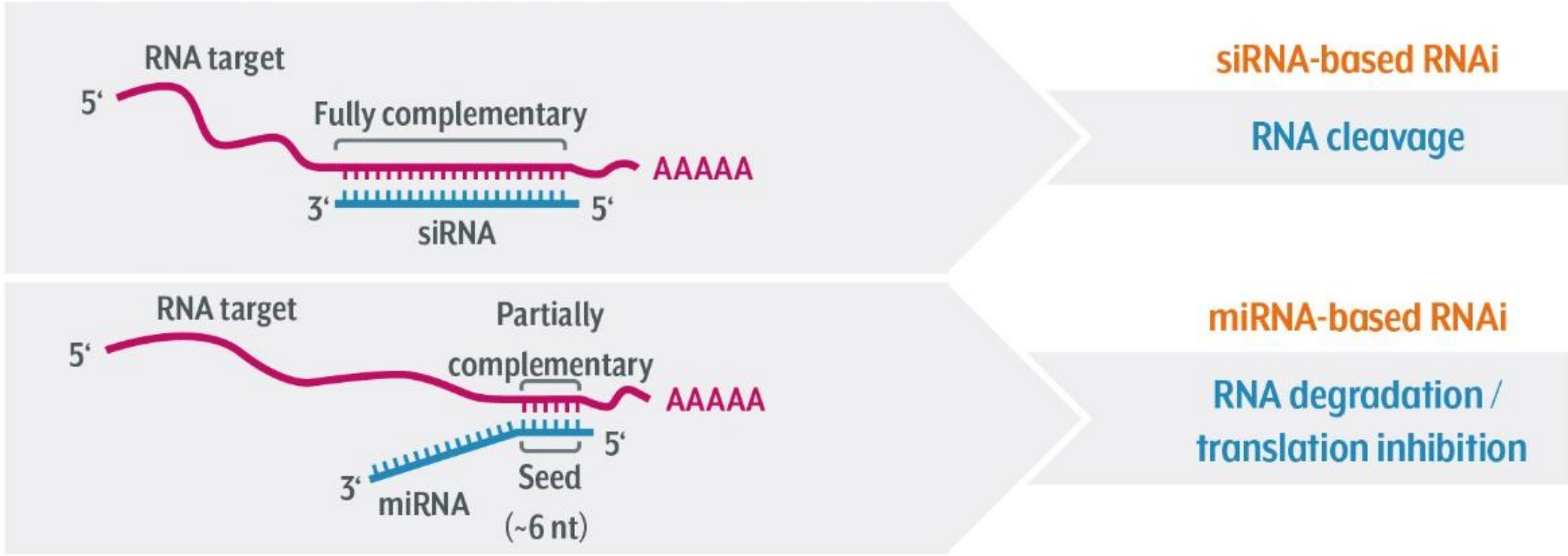


siRNA off-target effects are **wide-spread** and **siRNA concentration-dependent**

RNAi Drawback – Off-Target Effects

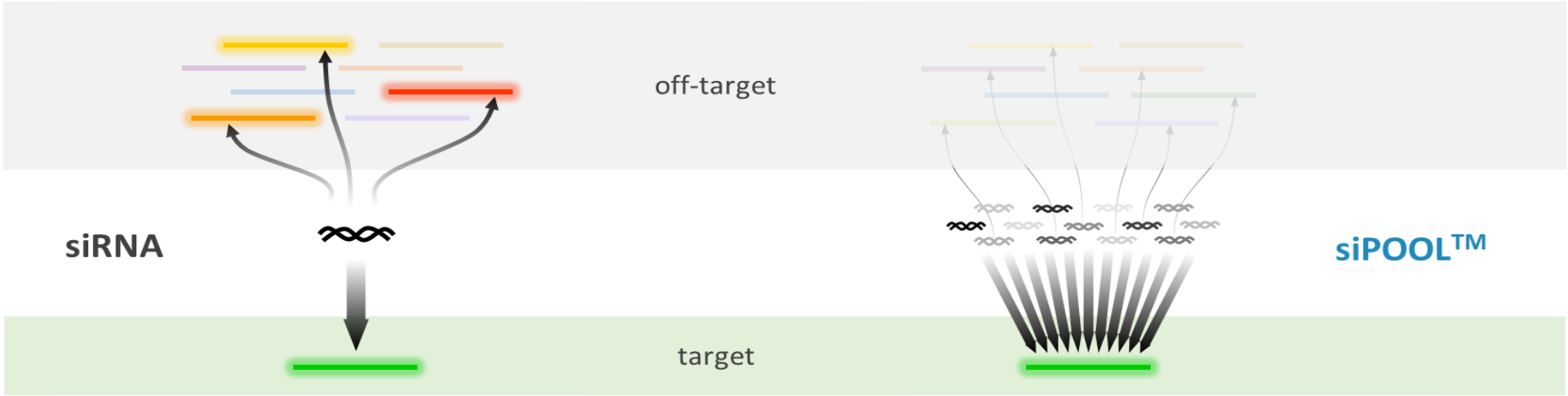
Mechanism

Leading cause: miRNA-like transcript downregulation



Off-targets are caused by siRNAs acting like miRNAs!

Power of Pooling



- **Multiple off-targets**
- **Low or variable efficiency**

- High target specificity
- **Increased efficiency & reproducibility**

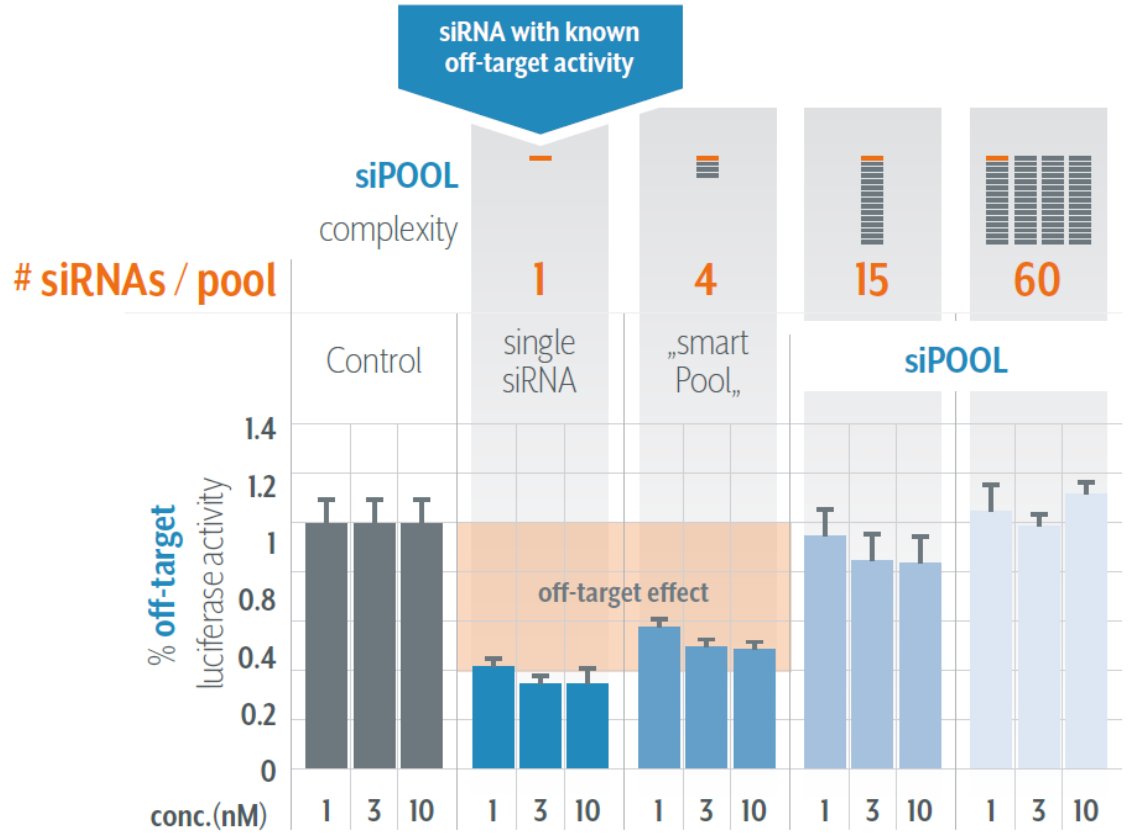
⇒**RNA interference (siPOOL™)**

⇒**RNA affinity purification (raPOOL™)**

⇒**Ribosomal RNA depletion (riboPOOL™)**

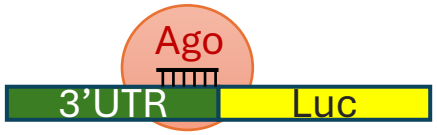
Why 30? Because 4 is not enough!

Off-target spiking experiment



Hannus et al., Nucleic Acids Res, 2014

Off-target luciferase reporter

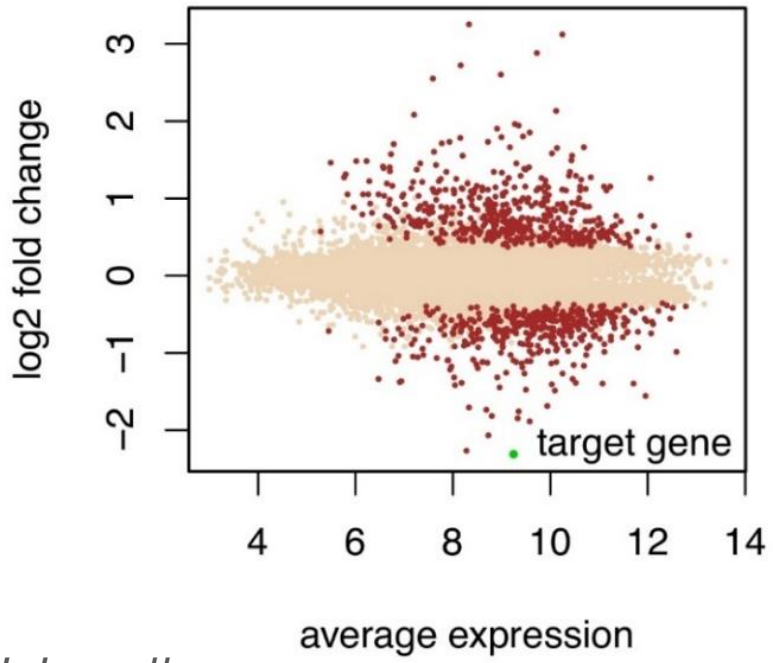


- *HeLa cells*
- *10 nM Scyl1 siRNA/siPOOL*
- *24-48 h*
- *reporter assay, RT-PCR, Western, functional assay*

High siRNA complexity (> 15 siRNAs) needed to robustly reduce off-target effects

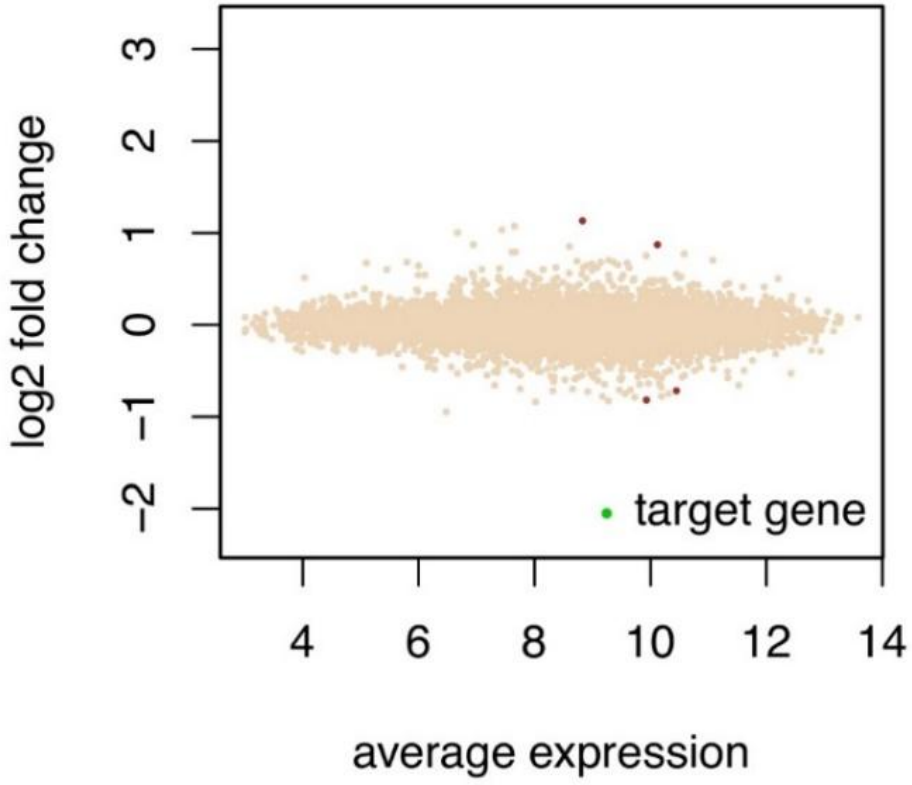
Increased Specificity with siPOOL™ for Gene Silencing

Single siRNA



siRNA within siPOOL

siPOOL



- *HeLa cells*
- *3 nM Scyl1 siRNA or siPOOL*
- *48 h*
- *whole transcriptome profiling by Affymetrix Microarray*

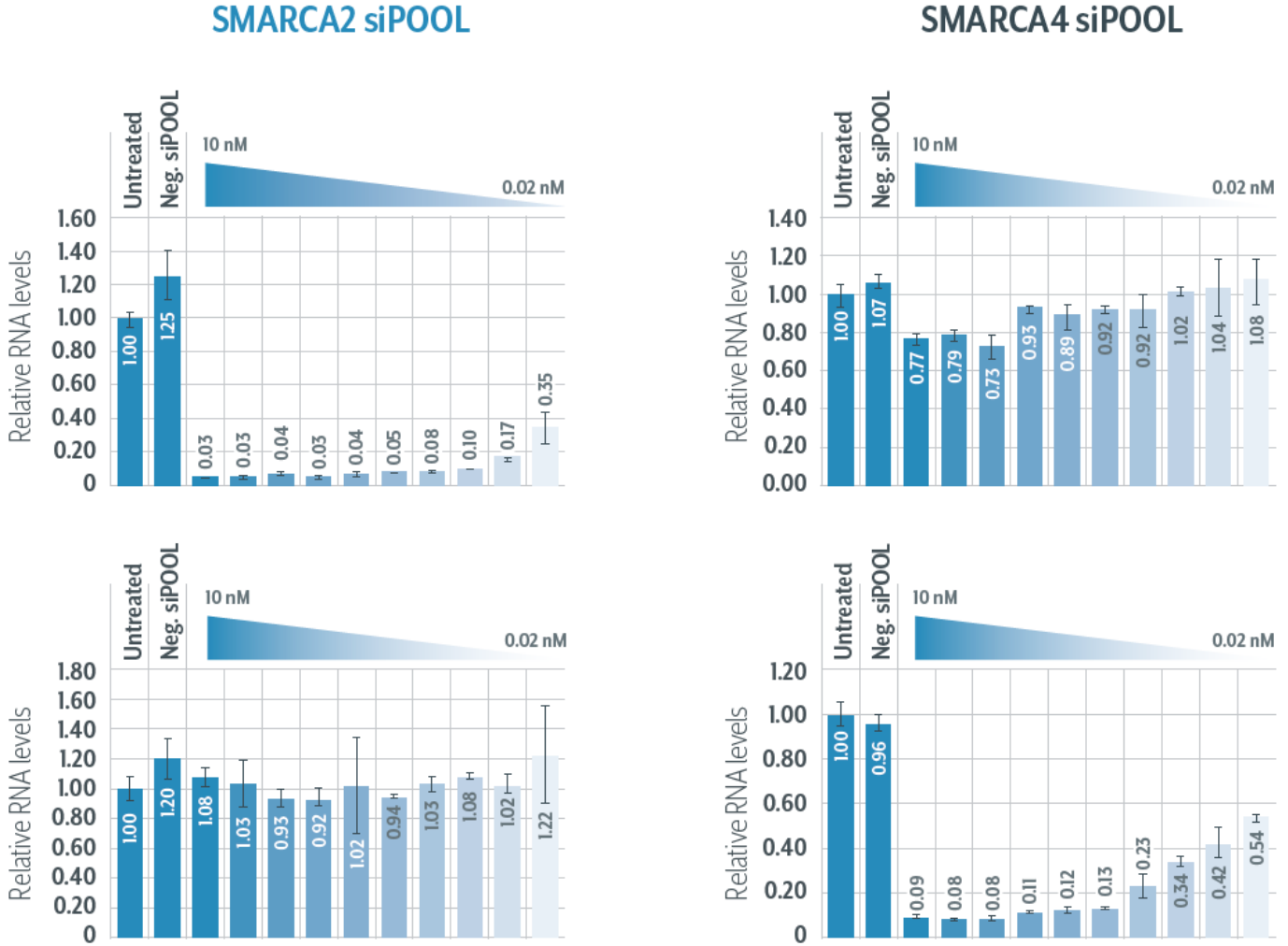
Hannus et al., Nucleic Acids Res, 2014

POOLing reduces off-target effects

Increased Efficiency with siPOOL™ for Gene Silencing

RT-PCR
SMARCA2

RT-PCR
SMARCA4



Dr. Mona Malz, PhD
Senior Scientist
Cancer Drug Discovery
German Cancer Research Center (DKFZ)
Heidelberg, Germany

POOLing increases transcript coverage and targeting efficiency

Specific and efficient targeting of long non-coding RNAs with siPOOLs

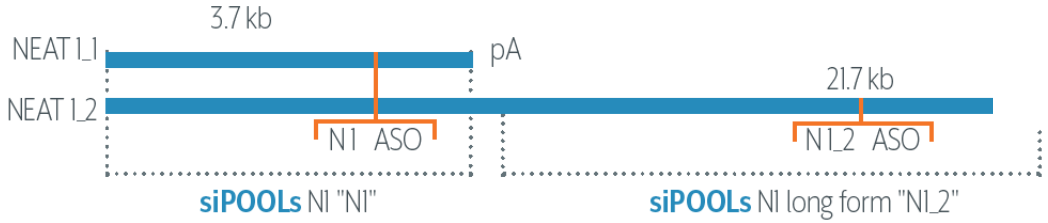
lncRNA targets

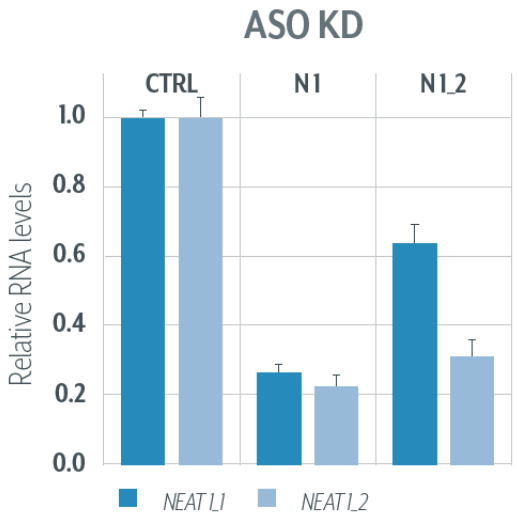
Ms. Jasmine Barra
PhD Student
Lab for Molecular Cancer Biology
Prof. Dr. Chris Marine Group
VIB Center for the Biology of Disease
KU Leuven, Belgium

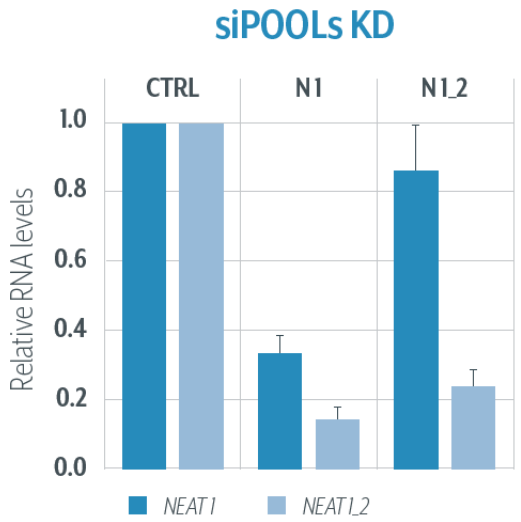
„For our research purpose the use of **siPOOLs** proved to be a key choice. We could overcome the major issues of both cell toxicity and off target effects we observed using GAPMERs.

In our hands the **siPOOLs** performed always with high reproducibility, allowing us to increase the efficiency of knock down of our target of interest, despite the poor results given by standard siRNA approaches.

Moreover siTOOLs could custom design for us isoform-specific **siPOOLs** that allowed us to address in a more specific way our biological questions."





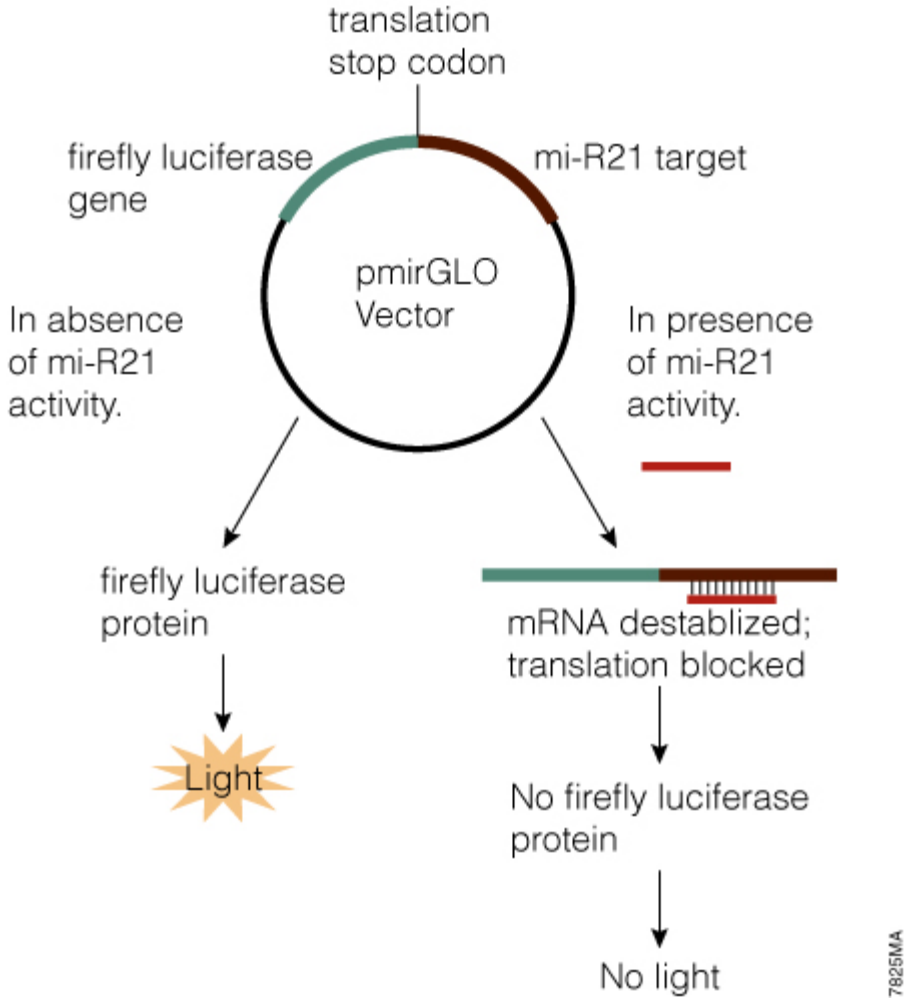


siPOOLs were used to knockdown long non-coding RNA, NEAT1, in MCF7 cells. An isoform-specific siPOOL (N1_2) was also generated. Both **siPOOLs** performed comparably with antisense oligos (ASO) and induced measurable phenotypic changes.

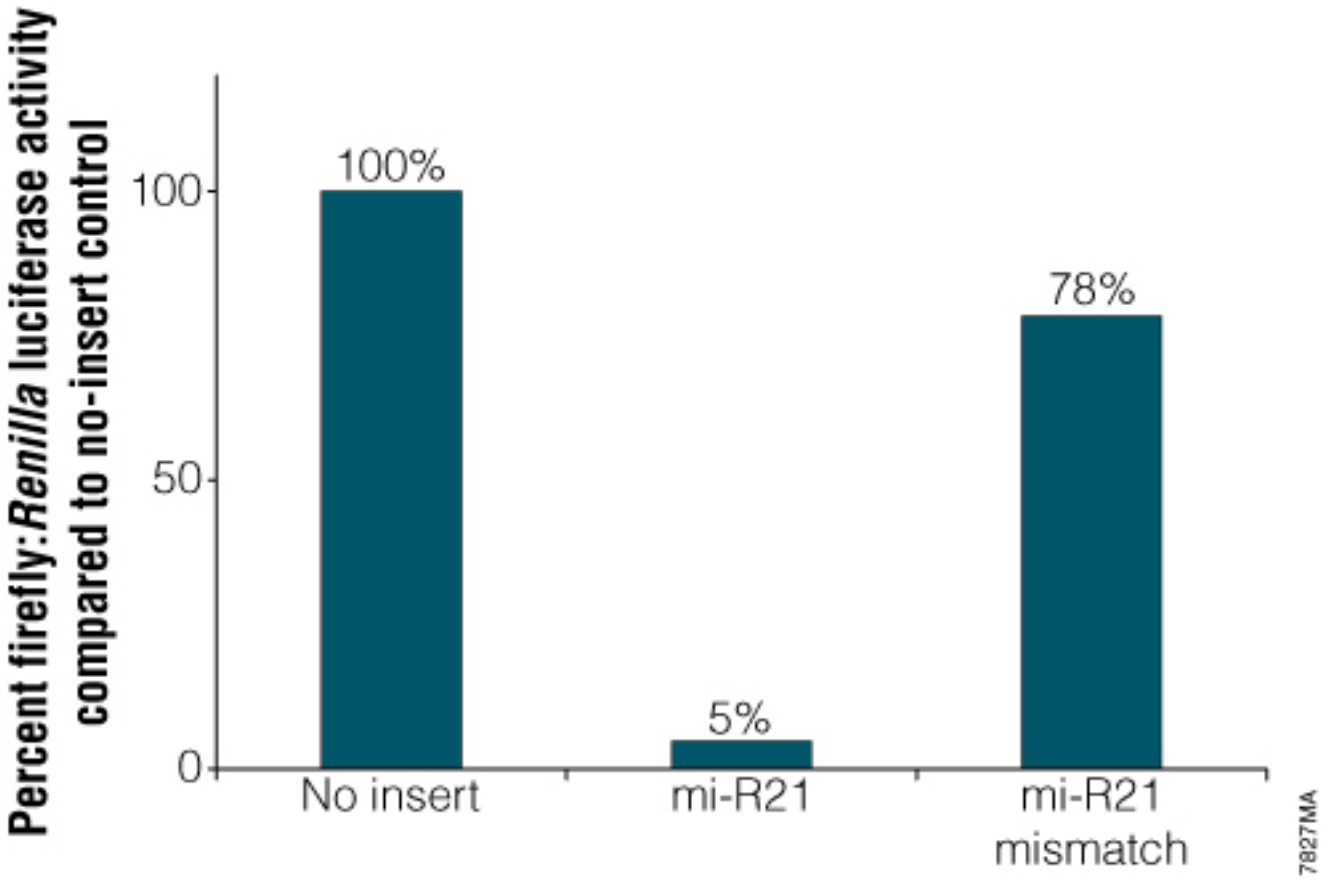
Data as published in Adriaens et. al, Nature Medicine, 2016[8]

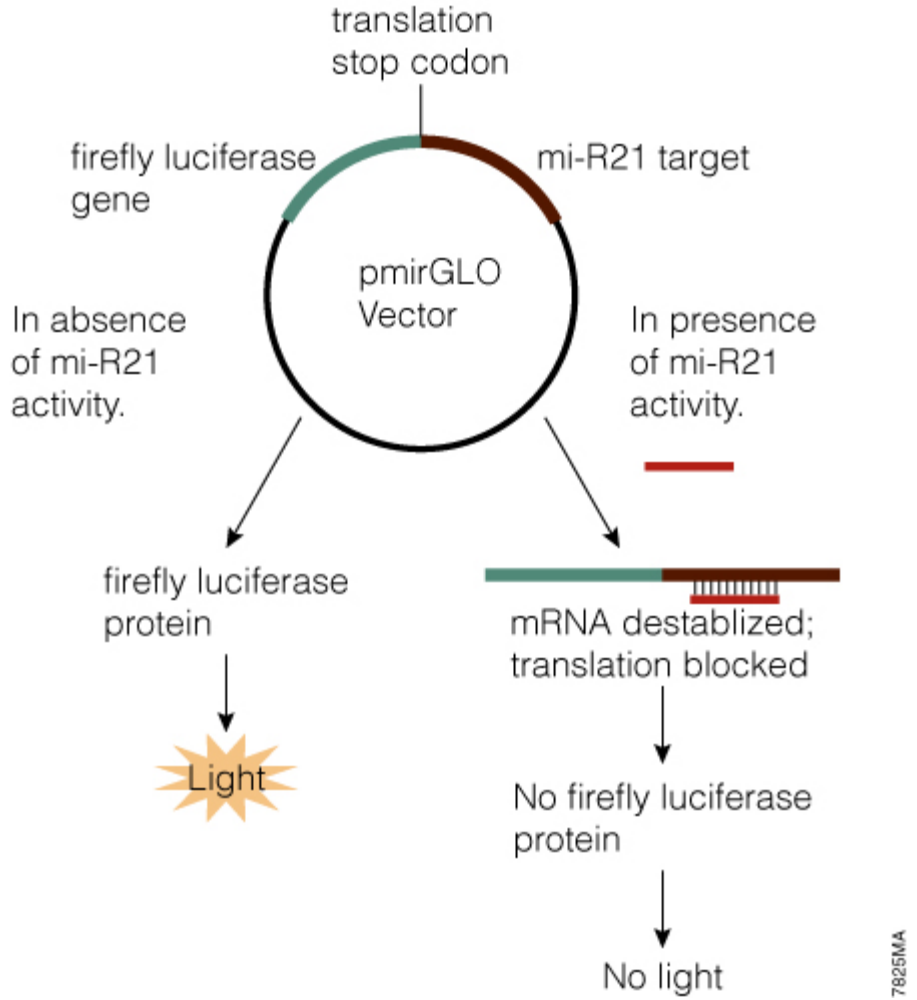
Evaluate microRNA Activity by Luciferase Assay

- Dual-luciferase vector for monitoring of miRNA binding
- Insert target sequence for the miRNA downstream of the firefly luciferase gene
- If added or endogeneous miRNA efficiently binds to the target sequence, firefly luciferase expression is reduced
- Renilla luciferase gene is used forsignal normalization



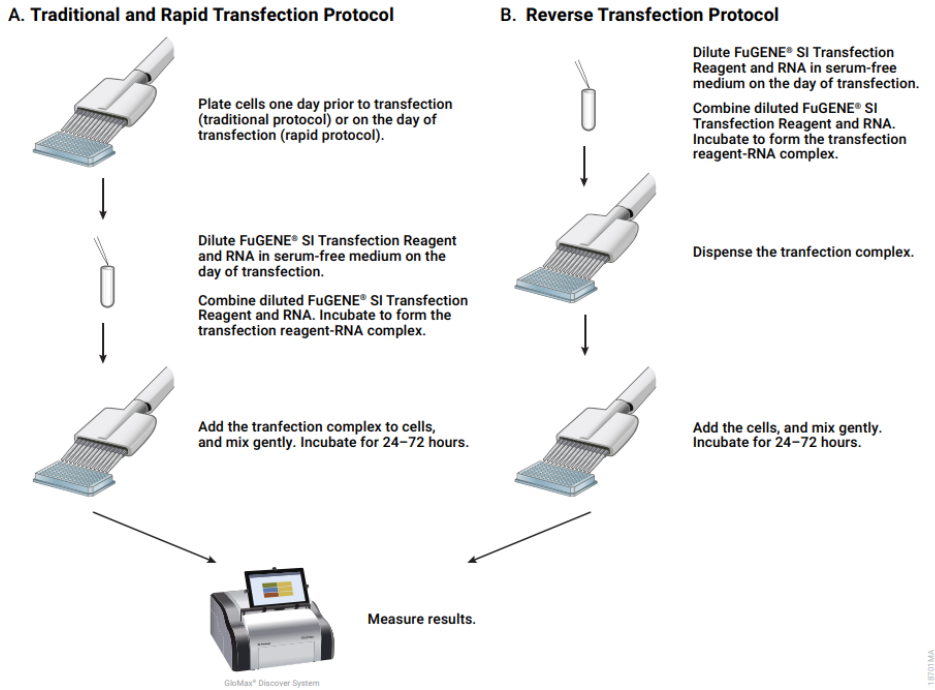
Evaluate microRNA Activity by Luciferase Assay

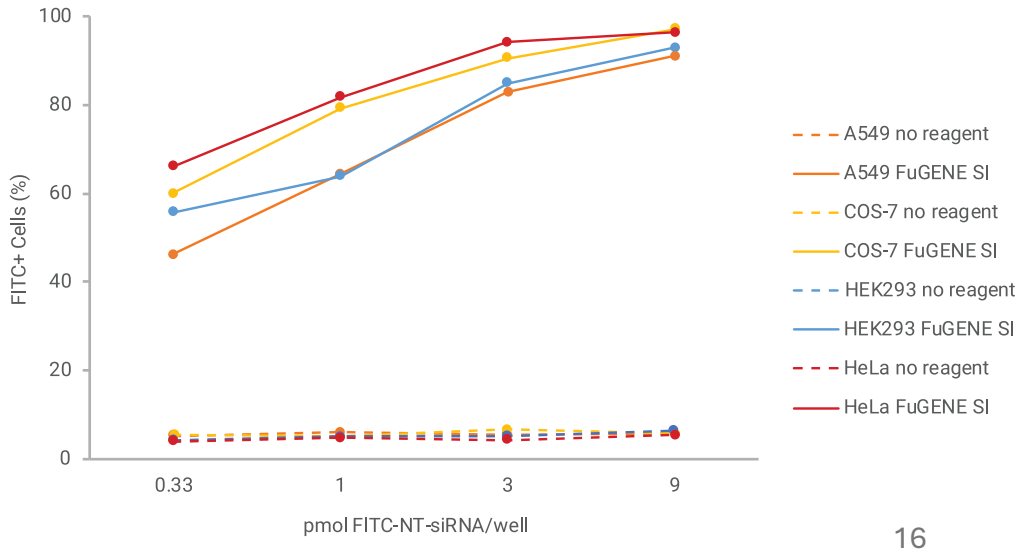




Fugene SI

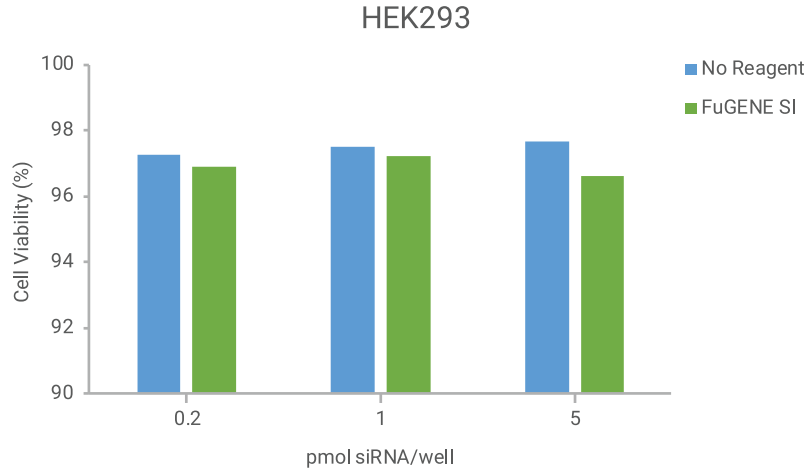
- **Versatile Transfection Reagent for siRNA, miRNA and Other Small RNA Molecules**
- High-efficiency transfection in challenging and routine eukaryotic cell lines
- Maximum knockdown with low toxicity—fewer cells required
- Simple protocol with minimal optimization
  - Mix the reagent with serum-free medium
  - Add RNA, mix and incubate for 5-15 min
  - Add the mixture to cells and incubate for 24-72 hours

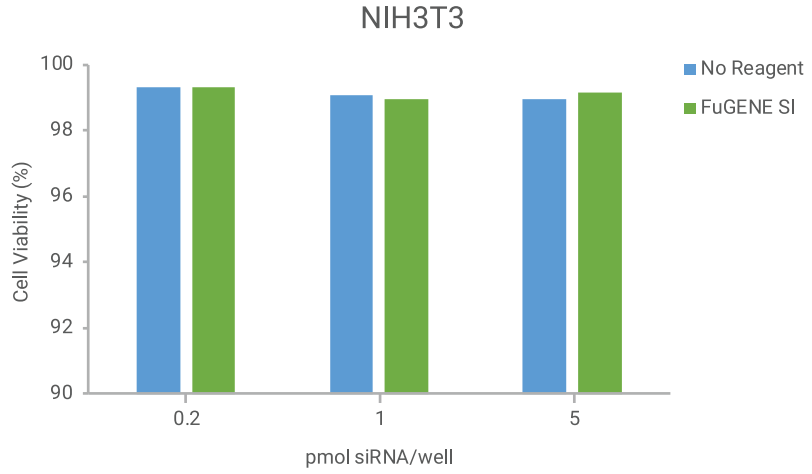


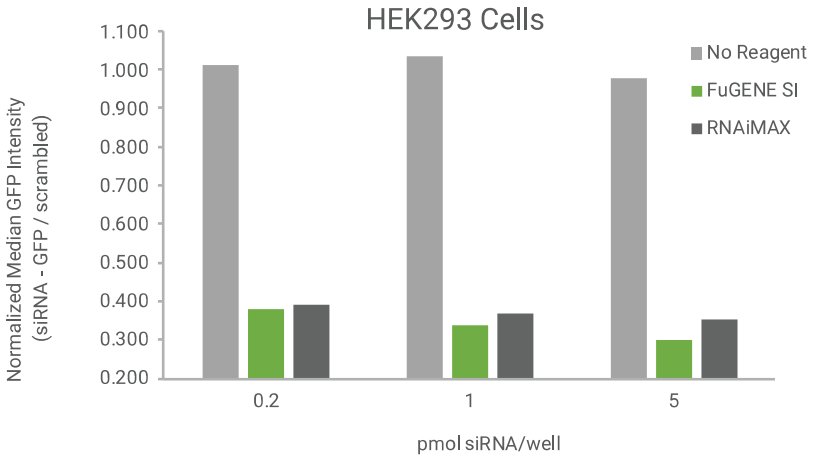


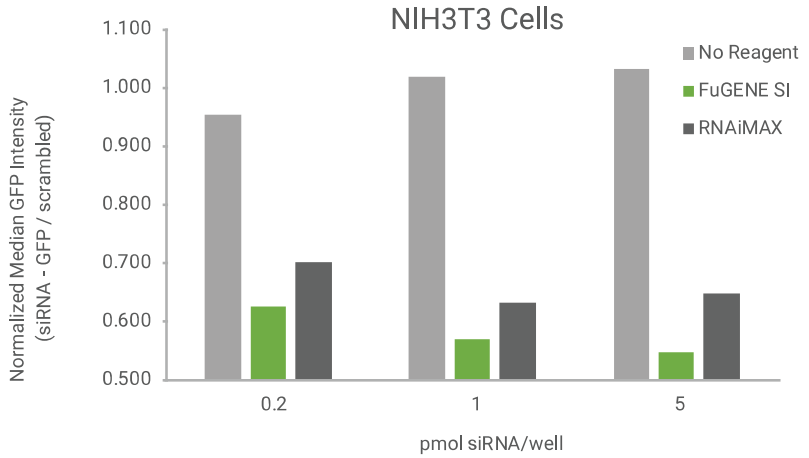
Efficient transfection of labeled small RNAs
Cell lines were plated in 96-well microplates and transfected with 0.4µl of FuGENE® SI or negative control and various amounts of FITC-labeled negative control siRNA. 24 hours post-transfection, the cells were analyzed via flow cytometry for FITC-positive signal.

Fugene SI – Efficient Knockdown, Low Toxicity







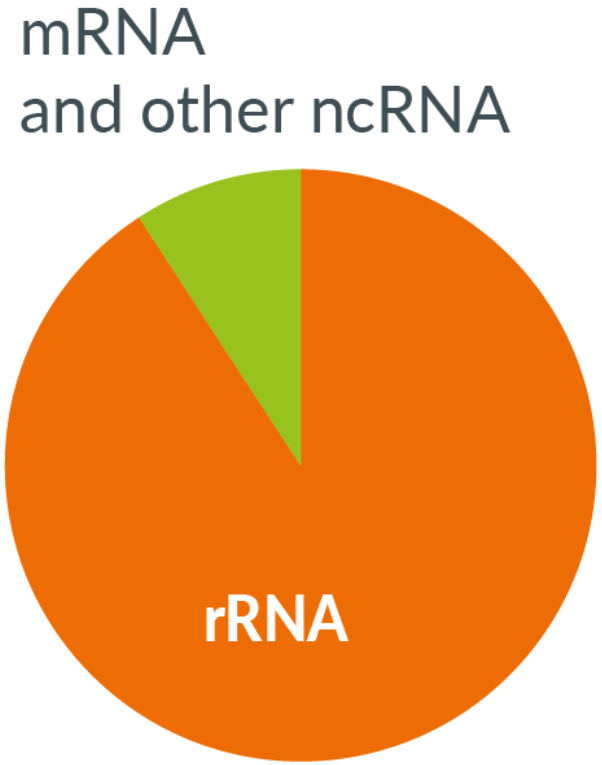


HEK293 & NIH3T3 cell lines expressing GFP (Cell Biolabs) were seeded in 96-well plates and transfected with 0.2, 1.0 or 5pmol of GFP targeting siRNA or negative control (Dharmacon™, Horizon) along with 0.3µl of FuGENE® SI reagent. Cells were analyzed via flow cytometry 48 hours post-transfection to measure cell viability and % of GFP knockdown efficiency.

Summary

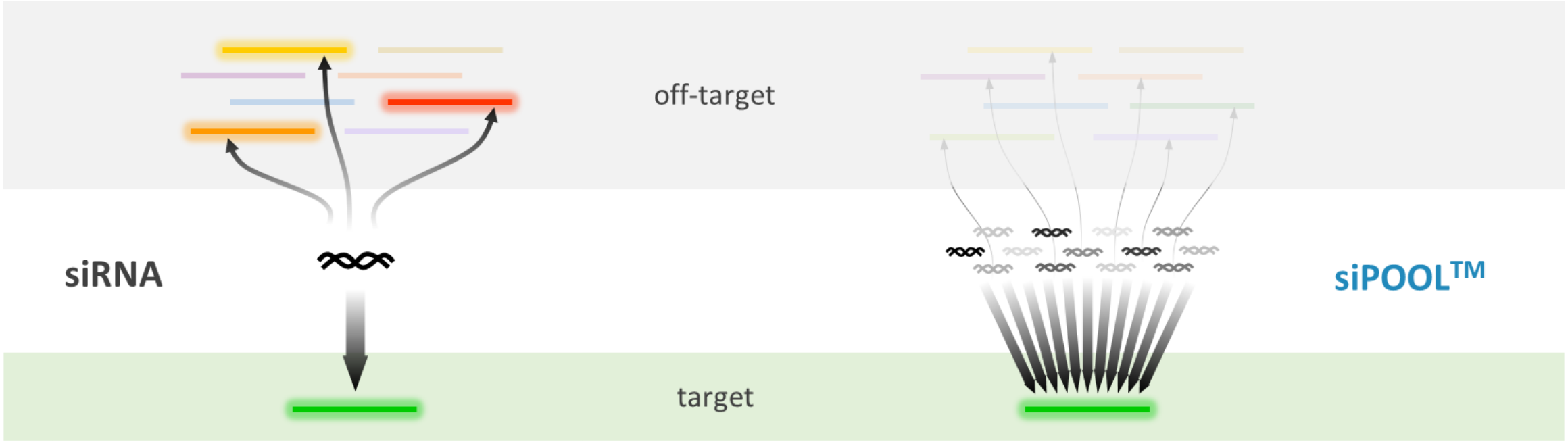
- The **dominance of siRNA off-target effects** produces variable results and hinders data interpretation
- **siPOOLs effectively counter siRNA off-targets** and improves reliability of RNAi experiments
- **Other advantages of siPOOLs**: open design support, quality production, excellent customer support
- **siPOOLs** can be prepared for any gene that has sequence in the NCBI database, just provide is with gene ID

Why do rRNA depletion?



- Ribosomal RNAs (rRNA): 80-90% of total RNA
- Limits detection of relevant RNAs – messenger RNA (mRNA) and non-coding RNA (ncRNA)
- Cost savings for RNA-Seq

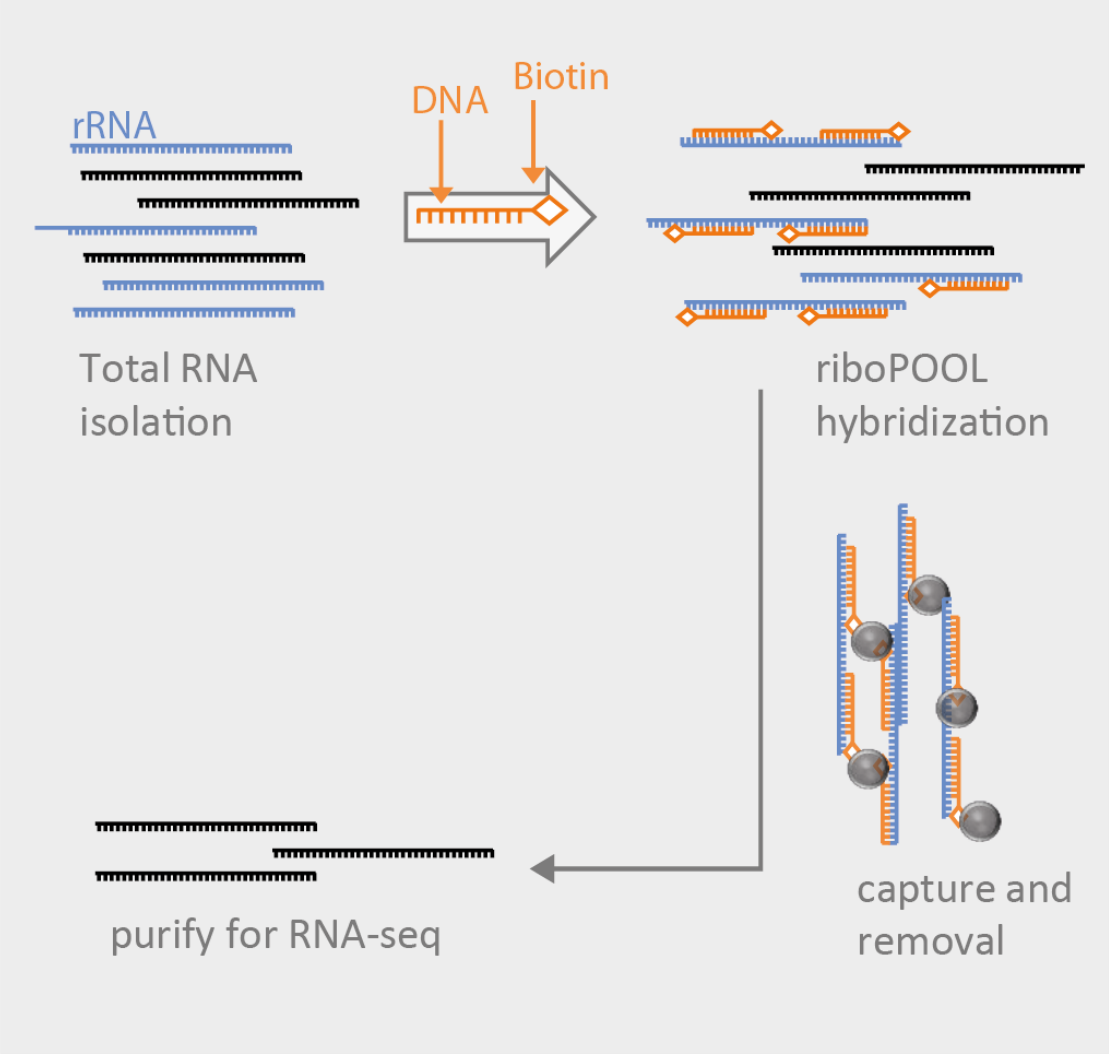
Pack Hunter Approach - Power of Pooling



- **Multiple off-targets**
- **Low or variable efficiency**
- **4-8 oligos at nanomolar concentration**

- High target specificity
- **Increased efficiency & reproducibility**
- **30 oligos at picomolar concentration (60-350 in riboPOOLs)**

rRNA depletion with riboPOOLs - Workflow



25 min **Hybridization**

riboPOOLs are resuspended and hybridized to DNA-free total RNA (input range: 100ng -5µg).

30 min **Capture & Removal**

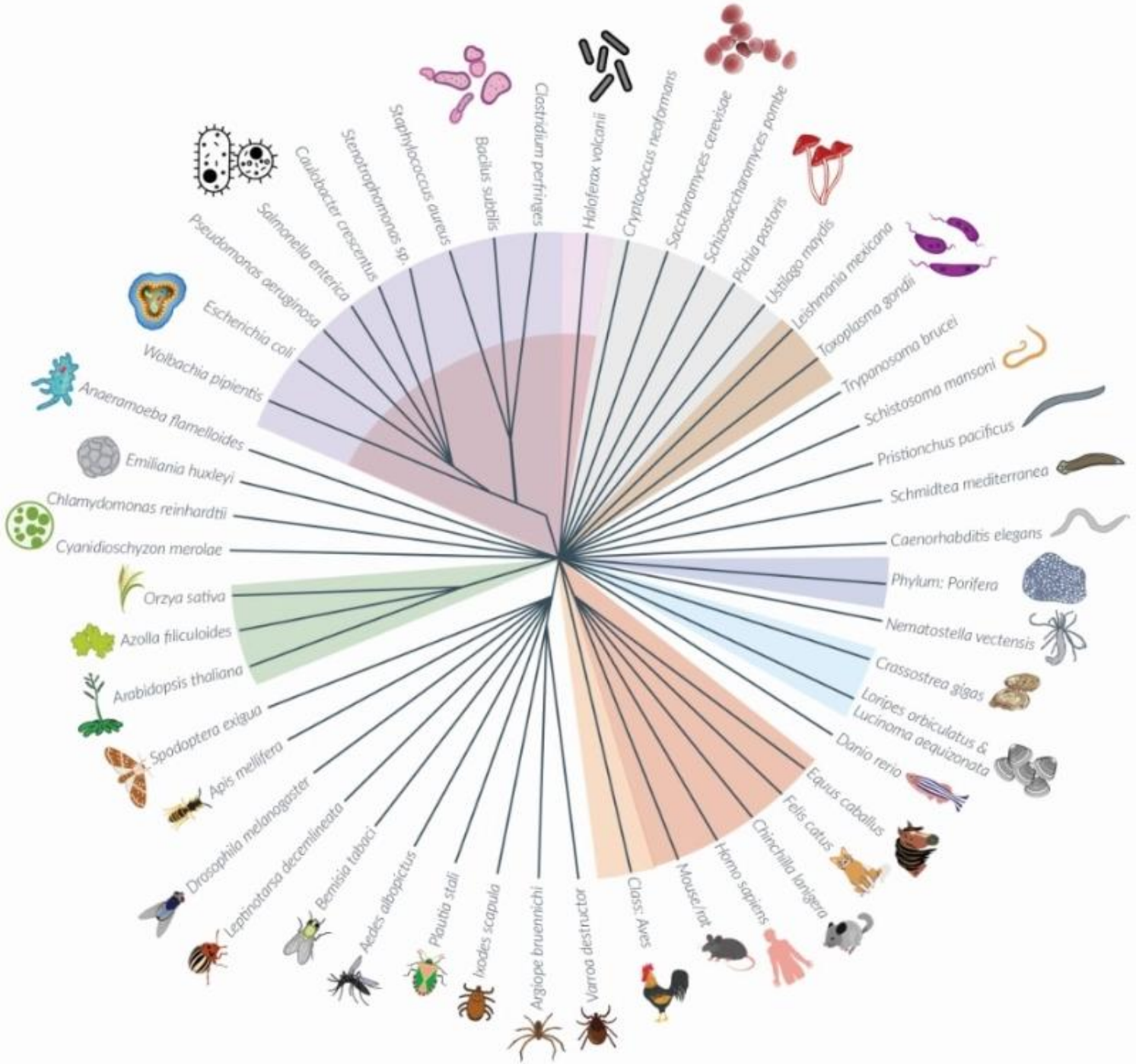
Streptavidin-coated magnetic beads separate riboPOOL-bound rRNAs.

15-90 min* **Purification**

Remaining relevant RNAs are purified by ethanol, silica column or SPRI beads prior to downstream analysis.

**Time required dependant on clean-up method*

- **Whole workflow done within 70 minutes**
- **Up to 98% rRNA removal**



multiple species riboPOOLs

Pan-riboPOOLs

Eukaryotes

Pan-Mammal riboPOOL
Blood Parasite riboPOOL
Pan-Fungi riboPOOL
Filamentous Fungi riboPOOL
Pan-Plant riboPOOL
Pan-Bird riboPOOL
Pan-Sponge riboPOOL

Prokaryotes

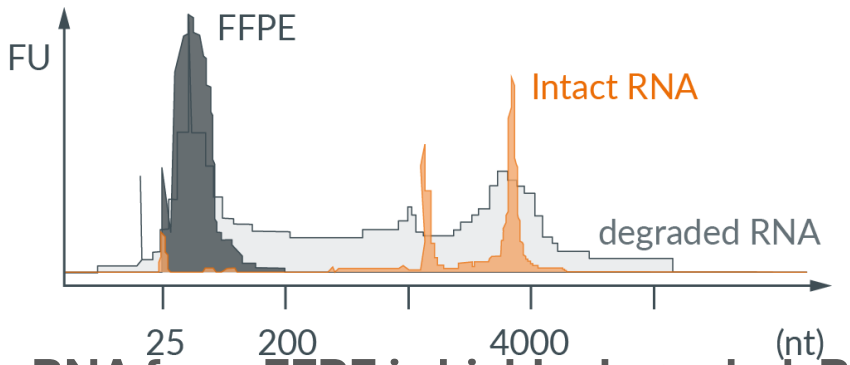
Pan-Prokaryote riboPOOL
Pan-Bacteria riboPOOL
Pan-Archaea riboPOOL
Pan-Actinobacteria riboPOOL

Species not listed? Create Custom riboPOOL with One-Time riboPOOL Setup Service

riboPOOLs for Any Species or Abundant RNA

degraded RNA

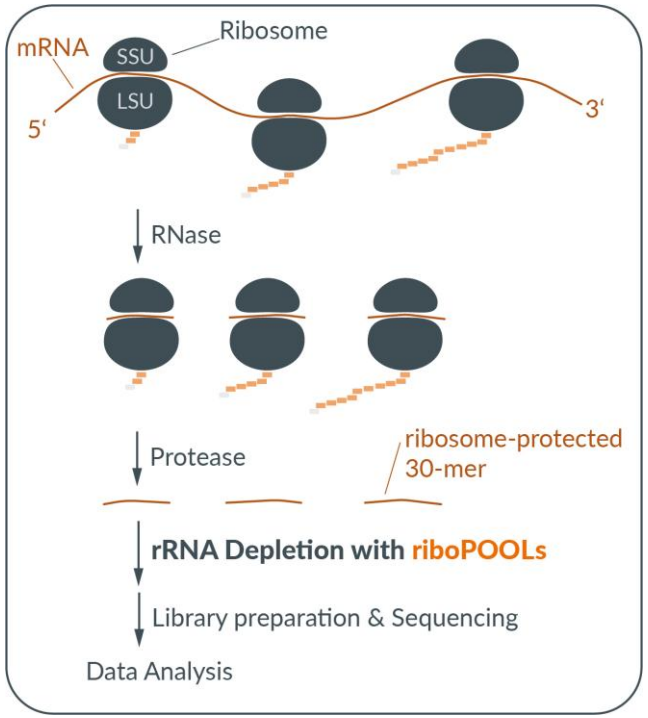
D. melanogaster degraded RNA riboPOOL
human FFPE/degraded RNA riboPOOL
human/mouse/rat FFPE/degraded RNA riboPOOL
mouse/rat FFPE/degraded RNA riboPOOL



- **RNA from FFPE is highly degraded, RIN values below 2**
- **Specifically designed tiled overlapping oligos covering the whole rRNA sequences**

ribosome Profiling

human Ribo-Seq riboPOOL
human/mouse/rat Ribo-Seq riboPOOL
mouse/rat Ribo-Seq riboPOOL
C. Elegans Ribo-Seq riboPOOL



- **Analysis of actively translated RNAs bound to ribosome**

other abundant RNAs

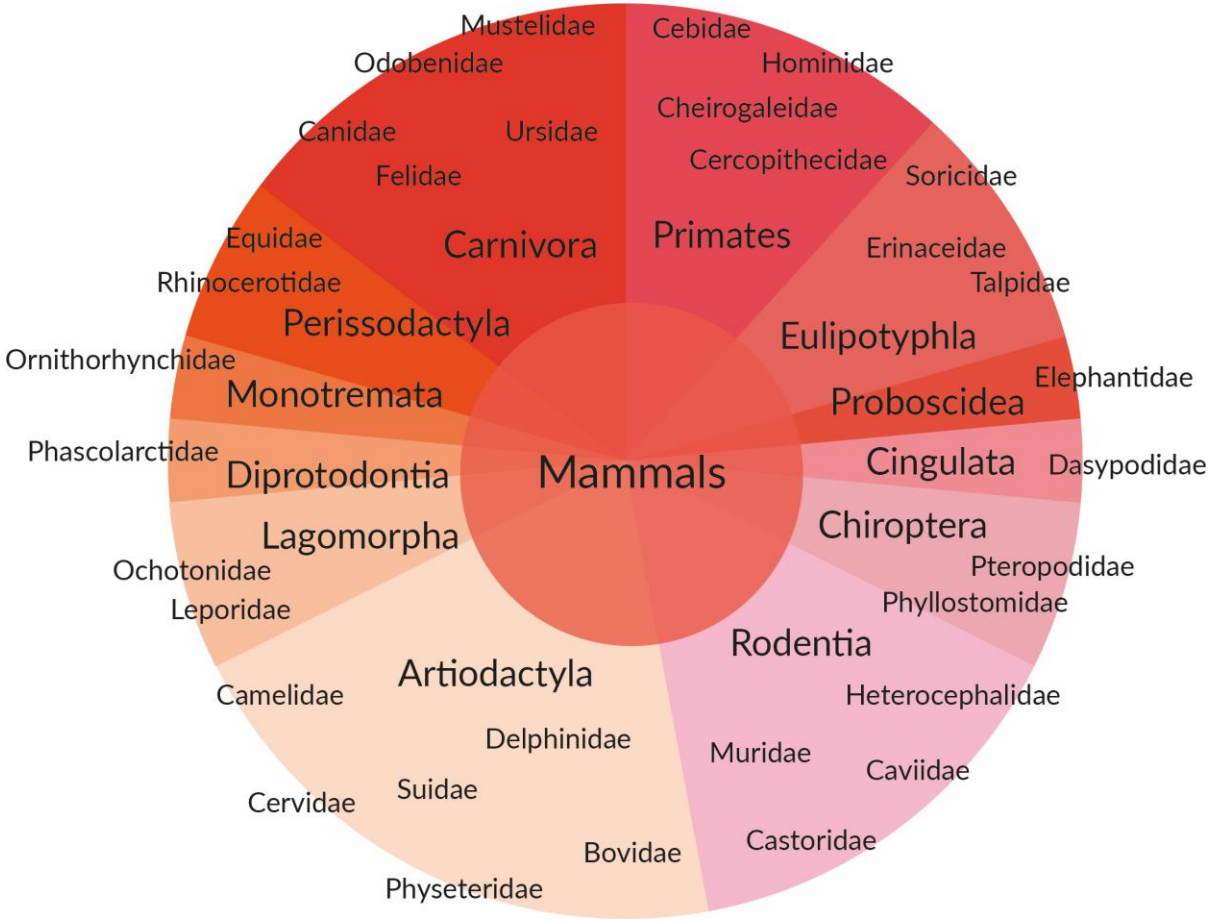
Human globin mRNA
Pola-A tailed RNAs (euk. mRNAs)
SARS-CoV-2 RNA

Combination riboPOOLs

Combine any read-made riboPOOL for single step depletion of mixed samples

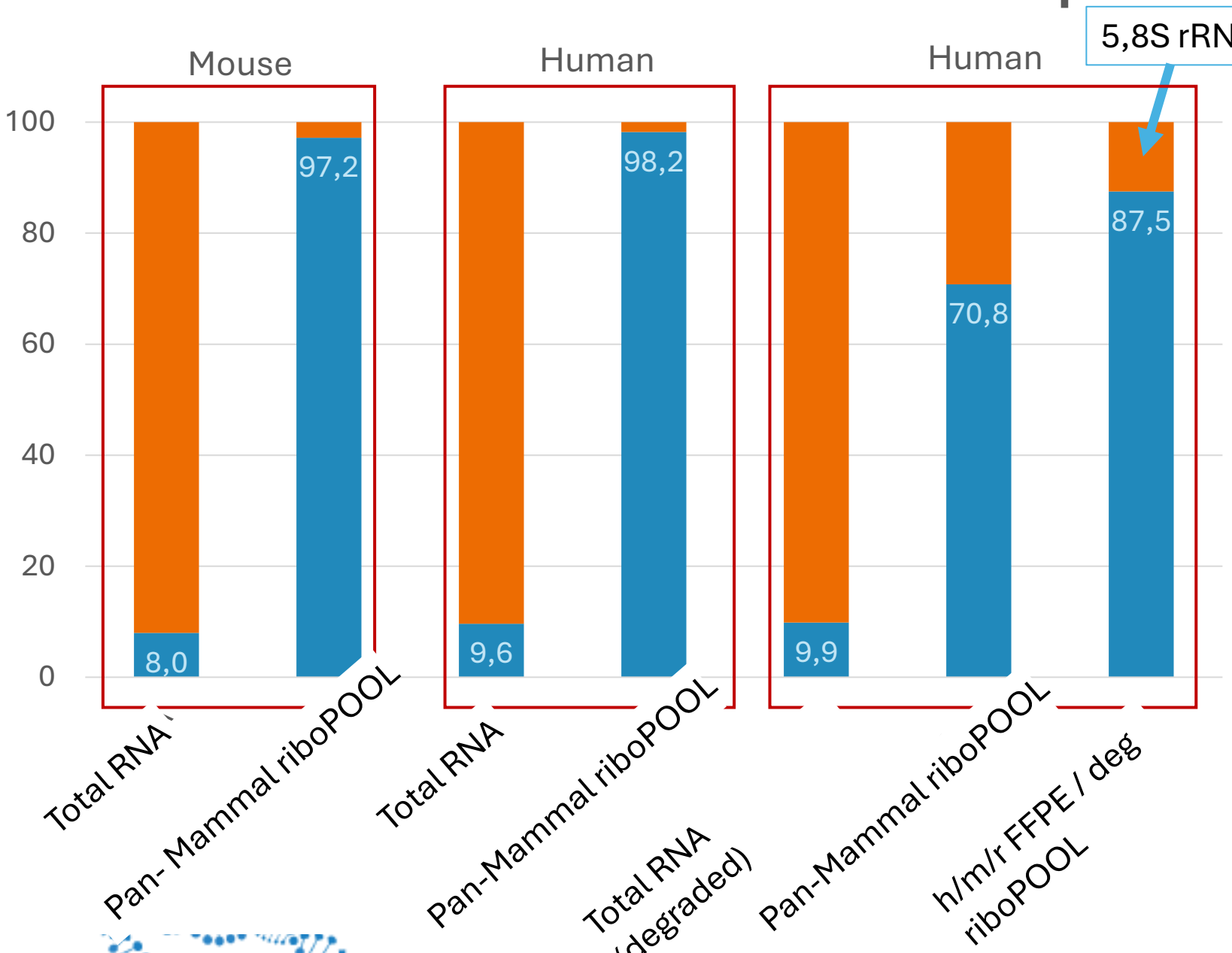
Species not listed?
Create Custom riboPOOL with One-Time riboPOOL Setup Service

Pan-Mammal riboPOOL for Universal Mammalian rRNA Depletion



- Efficient rRNA depletion tool
- Broad coverage of mammalia
- For tissue & cell culture derived RNA
- Targets 28S, 18S, 5.8S & 5S rRNA
- Targets mitochondrial rRNA

Pan-Mammal riboPOOL efficient across Species



Experiment Conditions	
Input (ng)	1000 of human and mouse RNA
RIN	~8
RNA Isolation	M&N
rRNA depletion	riboPOOL Kit
RNA clean-up	RNA XPClean (Beckman Coulter)
Library prep	NEB Kit (modified)
Sequencing	Illumina NovaSeq® 6000

Benefits of riboPOOLs

Complex pool & optimally designed oligos ensure high specificity and efficiency

- **Any species or abundant RNA**
- **Can detect small and long non-polyadenylated RNAs**
- **Suitable for metatranscriptomics**
- **Highly efficient and specific**
- **Broad RNA input range (10 ng – 3µg)**
- **Fast workflow**
- **Affordable**
- **HPLC purified**

riboPOOL Kit - Reagents up to Library Prep

- Available in 6 (trial), 12, 24 and 96 reaction sizes
- Shipped freeze-dried at room temperature
- Complete with buffers, beads, ethanol clean-up reagents
- Probes and beads alone also available

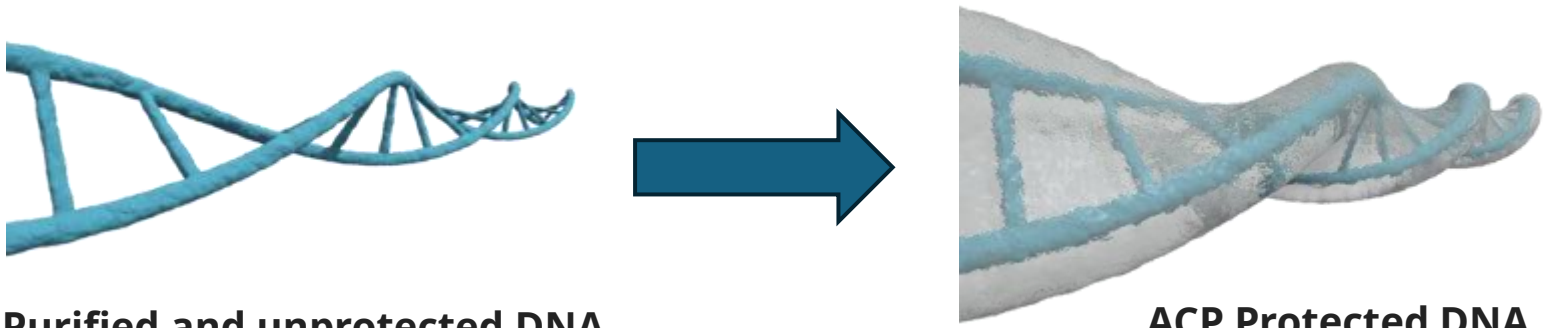
metabion

Quality to Trust

- German manufacturer of high-quality oligonucleotides
- Standard DNA/RNA oligonucleotides
- LNA/ZNA/PTO oligos and probes
- High-performance double quenched probes
- Dual-labeled probes
- siRNA/RNAi/dsRNA
- RNA Longmers
- NGS oligos
- M-Block long DNA oligos
- GMP manufacturing facilities for therapeutic oligos

GenTegra

- US manufacturer of long-term storage reagents for RNA and DNA
- Technology of active chemical protection
- Mix your sample with the storage reagent and dry
- Protective film is formed on the surface of nucleic acids preventing degradation by enzymes and oxidation

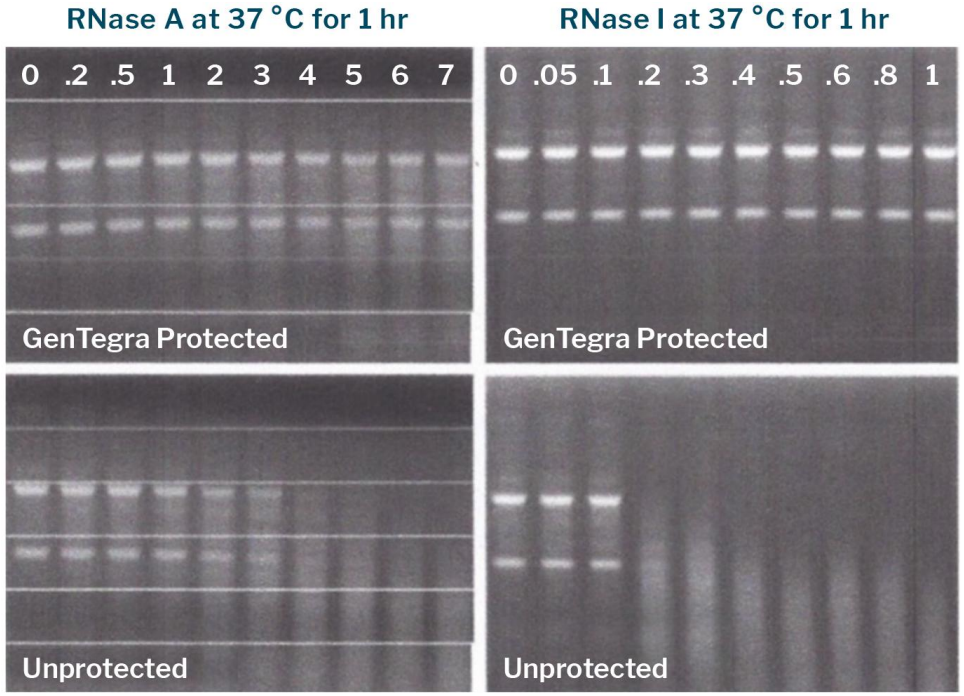


Purified and unprotected DNA

ACP Protected DNA

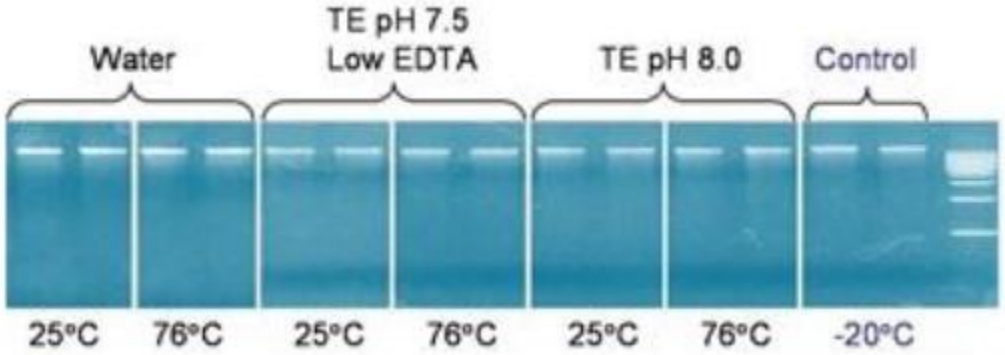
GenTegra

GenTegra RNA



HeLa cell RNA (5 µg) was incubated with the indicated amounts of RNase at 37 °C for one hour in the presence (top row) or absence (bottom row) of GenTegra RNAssure.

GenTegra DNA



Quality and integrity of DNA stored in GenTegraDNA Tubes is identical to DNA stored at -20°C. DNA was stored for 120 days at room temperature (25°C) or 76°C. 120 days of storage at 76°C is equivalent to 10 years of room temperature storage.

Thank you for your attention!

vojtech.ledvina@eastport.cz