RNA methods

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Outline

- **Working with RNA and extraction techniques**

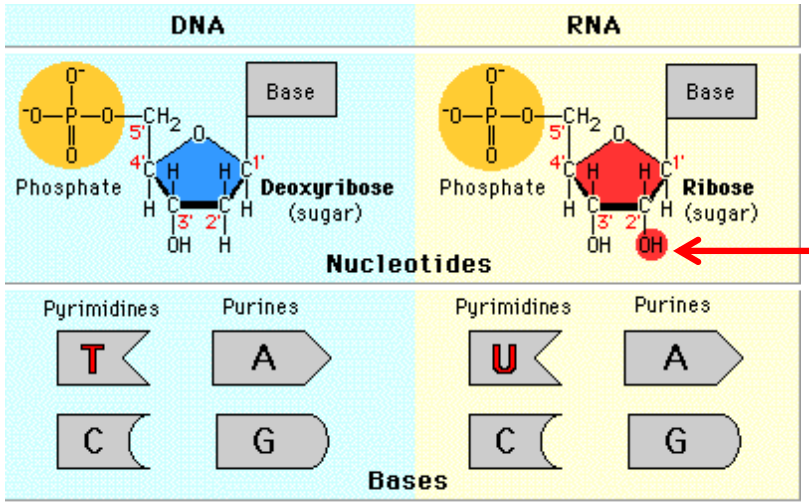
- **Detecting, quantifying and characterizing specific RNAs**
  - ***RT-qPCR***
  - ***Northern blots***
  - ***RNA FISH***

- **Detecting, quantifying and characterizing pools of different RNAs**
  - ***NanoString***
  - ***Microarrays***
  - ***RNA sequencing***

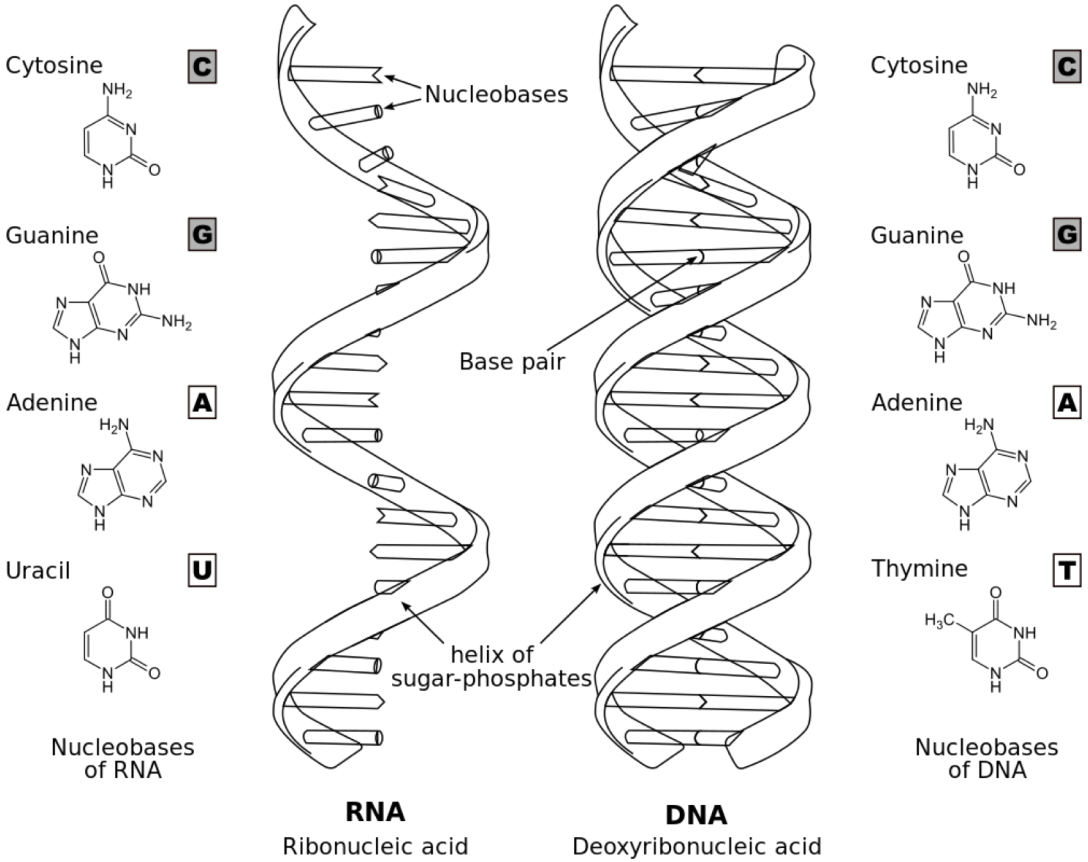
- **Studying RNA-protein interactions**
  - ***CLIP***
  - ***RNA pull-downs***

- **RNAi**
  - ***siRNAs***
  - ***shRNAs***

RNA *vs.* DNA



Increased chemical reactivity
➔ degrades easily in the laboratory



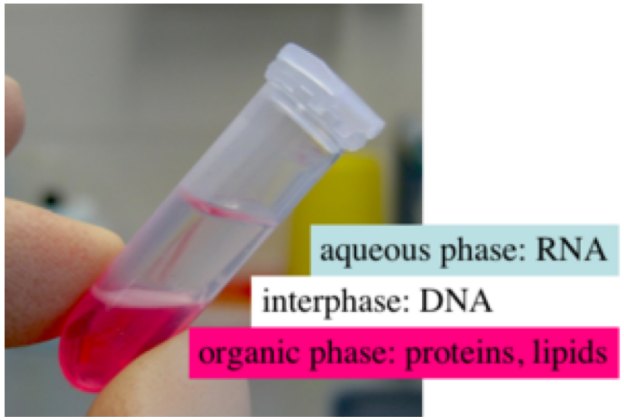
Working with RNA

- soak tube racks, gel trays, combs, etc. in 1% SDS o/n and then rinse with water and 70% EtOH
- clean bench with 1% SDS and then with water
- keep RNA samples always on ice
- avoid repeated freeze-thawing, store aliquots @ -80
- always wear gloves (and change gloves often); facemasks provide extra protection
- use filter tips (reserving a brand new set of pipettes for RNA work is a good idea)
- buy RNase-free water
- avoid equipment used for minipreps (centrifuges!) and cell cycle analysis
- never do plasmid preps and RNA work simultaneously

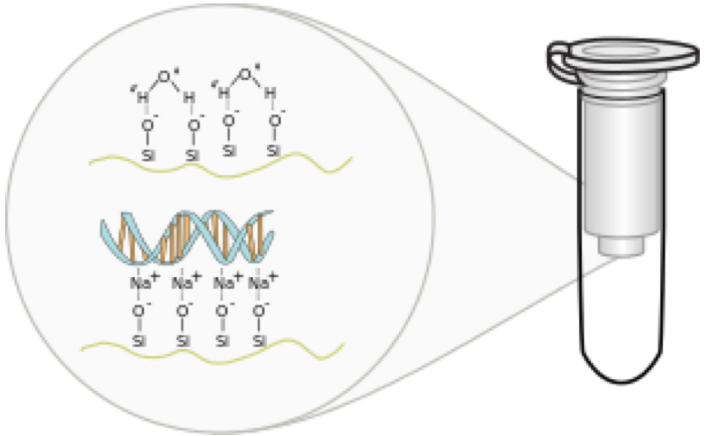
benchkote

Extraction of RNA from mammalian cells

- **Guanidinium thiocyanate-phenol-chloroform extraction ("TRIZOL"):**
  - contains phenol & guanidine isothiocyanate
  - disrupts cells and inactivates RNases
  - separates into aqueous and organic phases upon chloroform addition
  - RNA is precipitated from aqueous phase
  - pros: extracts RNAs of all sizes (but: extraction bias for some miRNAs, PMID 22749402)
  - cons: contains phenol; DNase digest required for RT Q-PCR and other applications



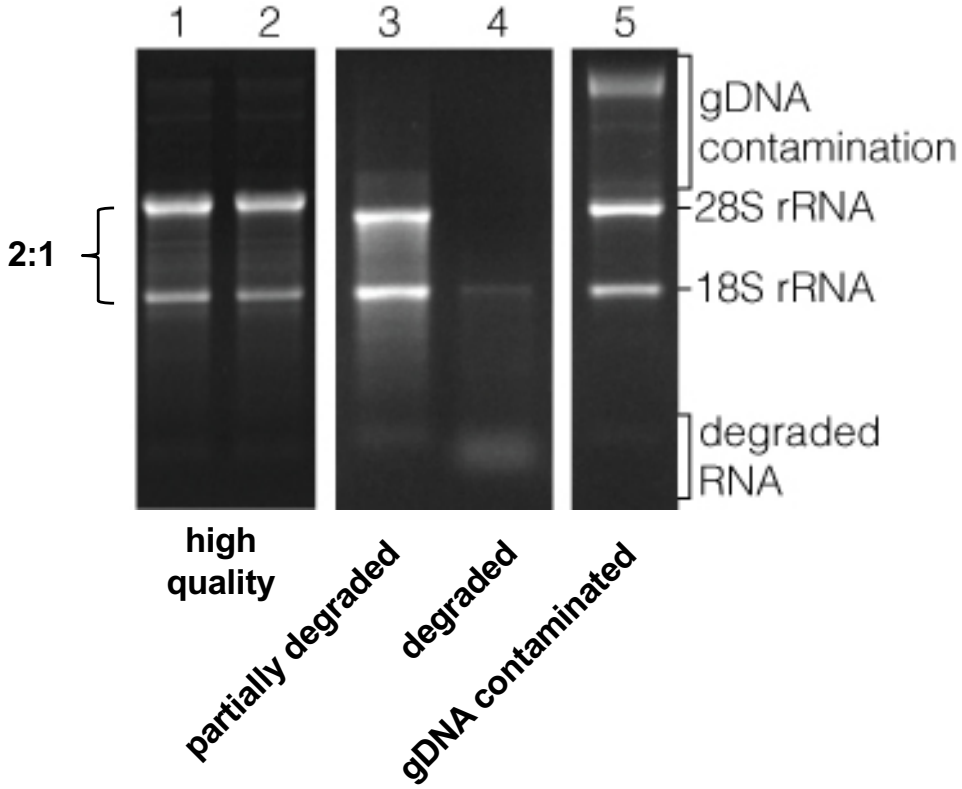
- **spin column-based RNA purification ("RNeasy"):**
  - disruption of cells in guanidine thiocyanate containing buffer
  - binding of RNA to a silica-based column
  - elution of RNA after washing
  - pros: fast on-column DNase digest
  - cons: only purifies RNAs of >200 nt (but: specialized miRNA extraction kits available)



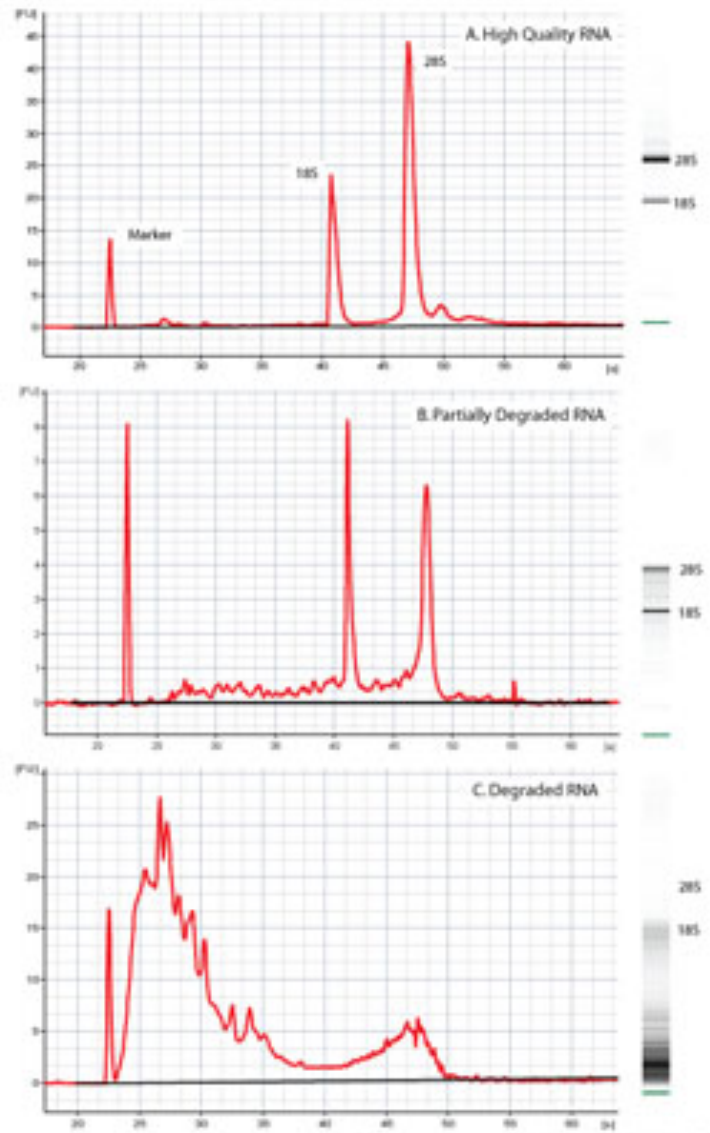
Quality control of total RNA samples

nanodrop: A_{260}/A_{280} ~ 2.0
A_{260}/A_{230} ~ 2.0-2.2

agarose gel:



bioanalyzer:



Detecting, quantifying and characterizing specific RNAs

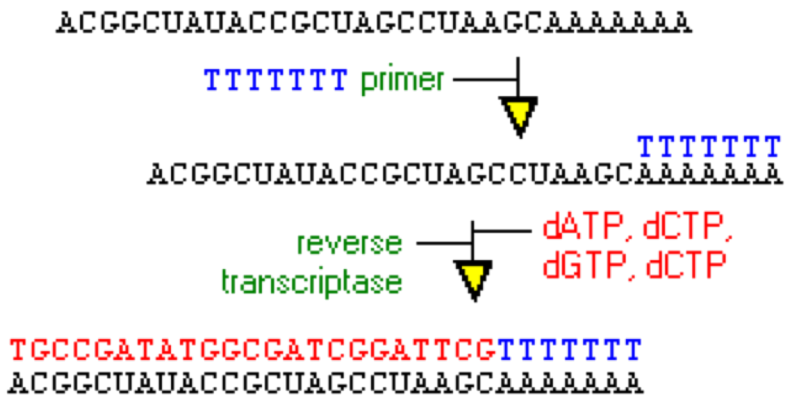
Reverse transcription

reverse transcription: conversion of RNA to cDNA

PRIMER:
- **random hexamers**
- **oligo(dT): typically $T_{12\text{-}19}$ – binds poly(A) tail on mRNA**

REACTION:
- **denature RNA and primer at 65–75C for 5 minutes, put on ice**
- **add buffer, dNTPs and reverse transcriptase enzyme**
- **ribonuclease inhibitor (e.g. RNasin or RNaseOUT) helps keep RNA intact**
- **incubate at 37-42C for 1–2 hours, heat inactivate**

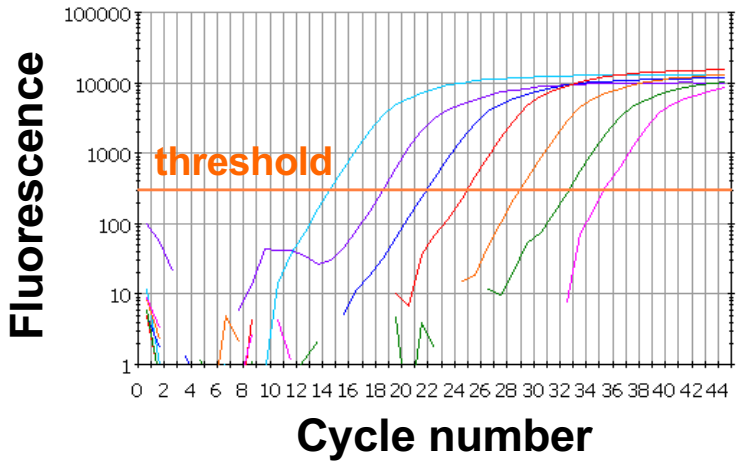
PCR:
- **use gene specific primers**
- **number of cycles depends upon abundance of target RNA**
- **NOT quantitative if used as an assay of mRNA levels**



Quantitative Real-Time PCR

A method of 'watching' the PCR as it progresses – allows quantification

- **Real-time PCR relies on detecting the PCR product as it accumulates during the reaction**
- **Detection is based on fluorescence**



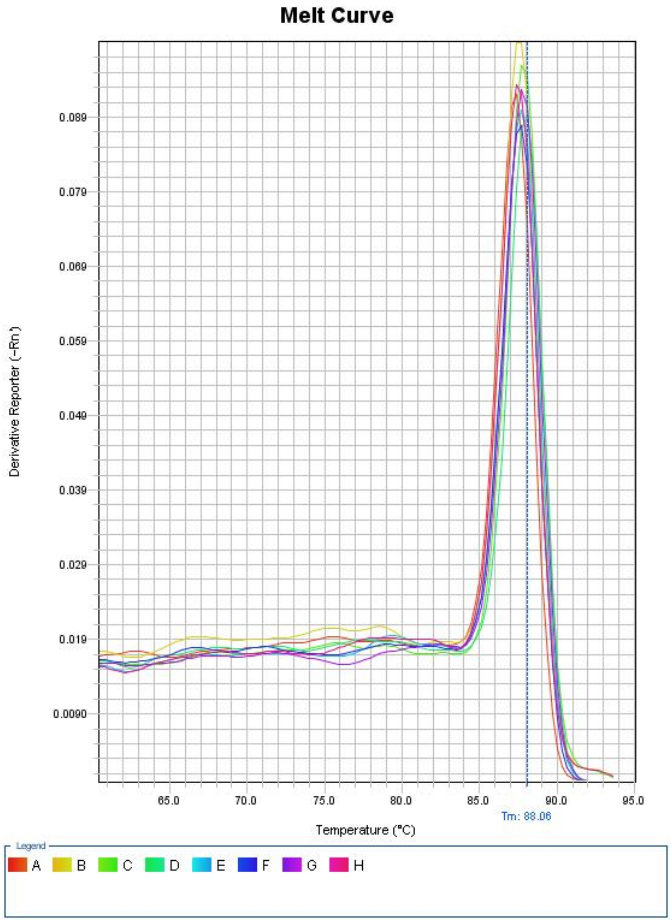
1) Non-specific intercalating dyes (e.g. SYBR green)
simply detect increasing amounts of double-stranded DNA accumulating

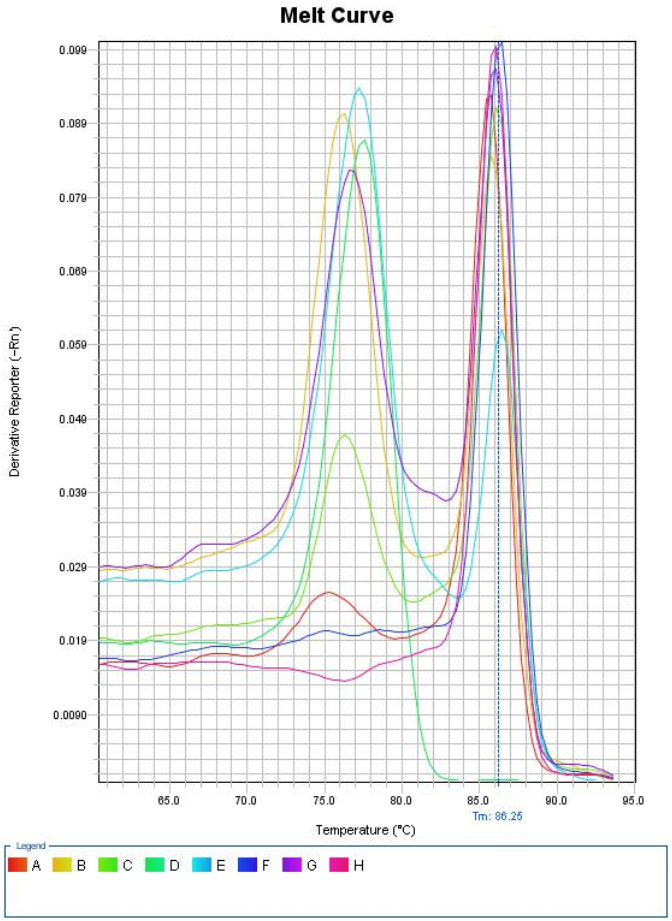
2) Fluorescent probes specific for the target of interest (e.g. TaqMan probes)

Non-specific intercalating dyes

- need to ensure that the reaction is specific (run out product at the end / run melt-curves / clone & sequence product)

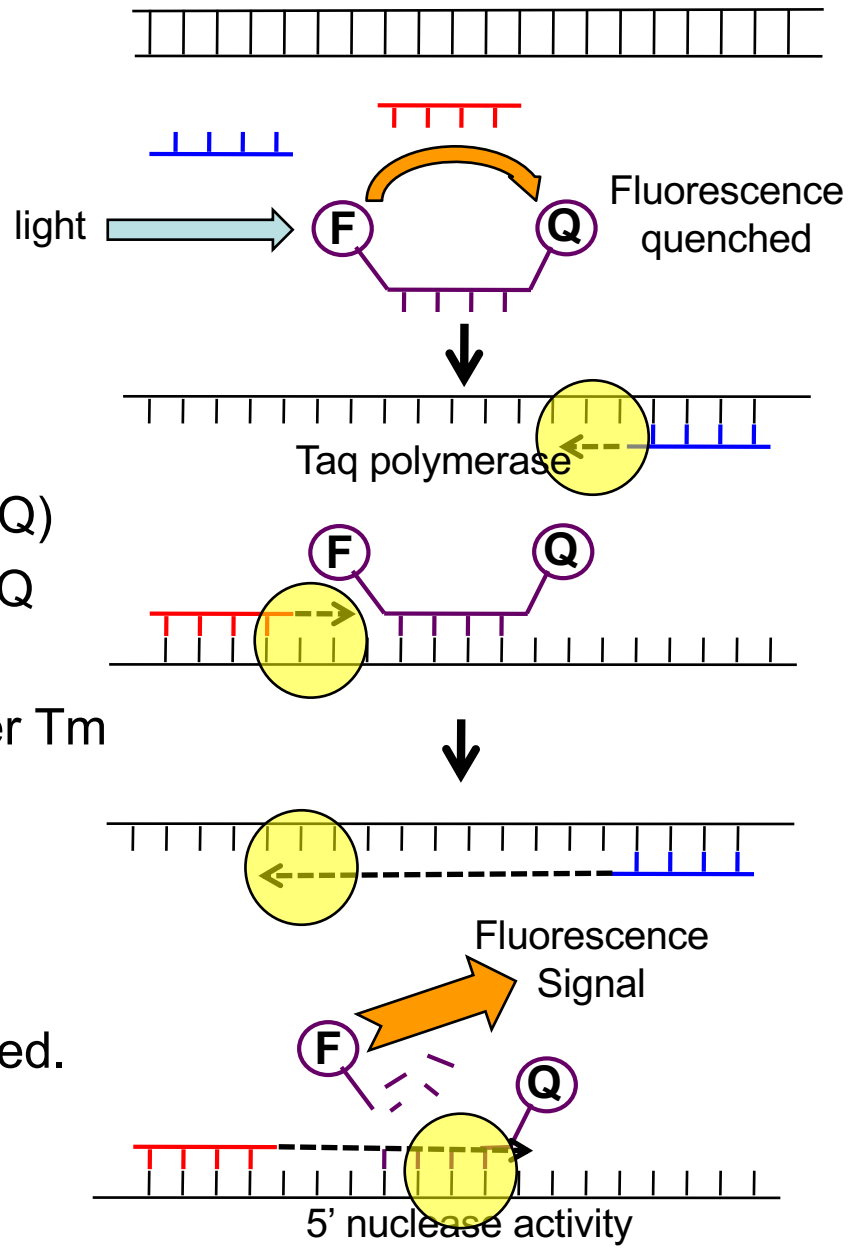
- Melt-curve: sharp single peak indicates specific amplification
  diffuse / multiple peaks demonstrate non-specific amplification



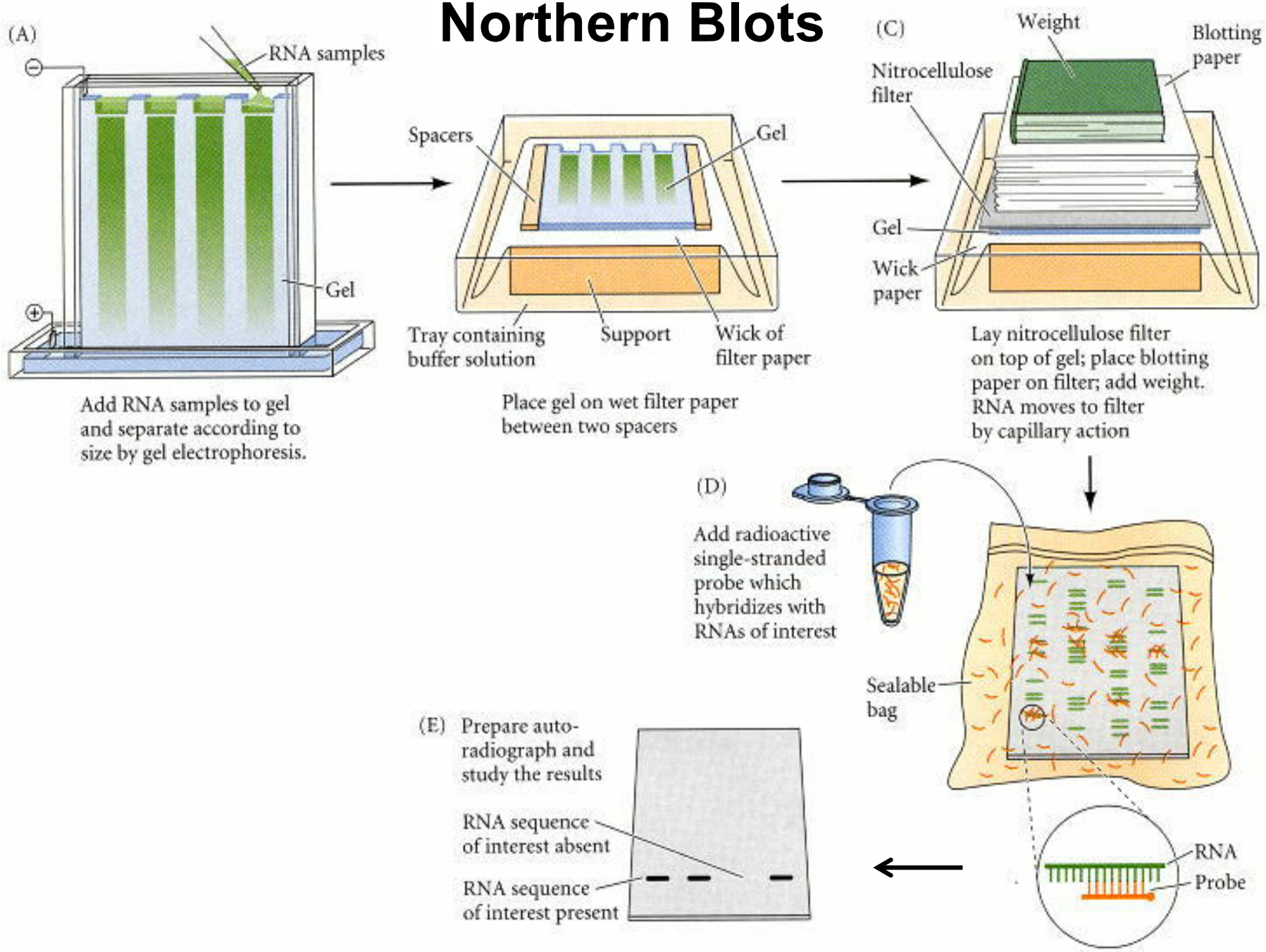


TaqMan probes

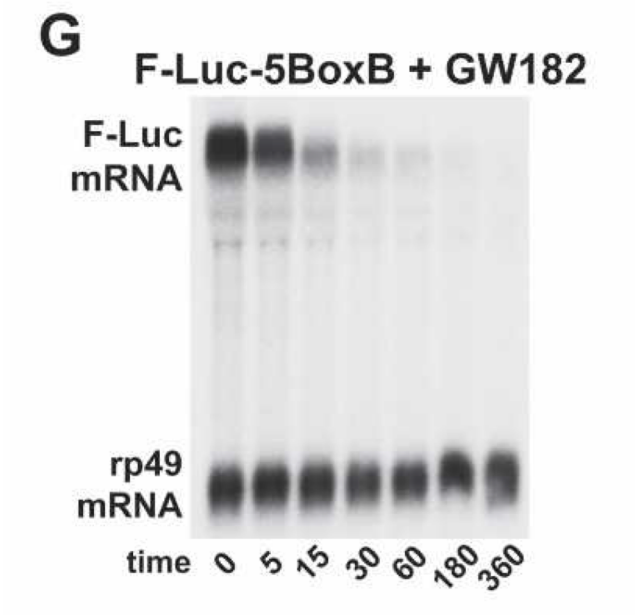
- Included in the PCR reaction:
  - Specific forward and reverse primers.
  - Probe specific for a sequence within the PCR amplicon.
- The TaqMan probe:
  - Fluorescent reporter dye (e.g. FAM, VIC, NED)
  - Quencher dye – often non-fluorescent (NFQ)
  - When linked on the same intact probe, NFQ quenches reporter dye fluorescence (FRET).
  - Probe Tm approx 8-10ºC higher than primer Tm
- Generating a signal:
  - 5’ exonuclease activity of Taq polymerase displaces and cleaves probe, releasing reporter dye from quencher – FRET disabled.
  - 1 molecule released per amplicon.
  - Signal increases as PCR progresses.

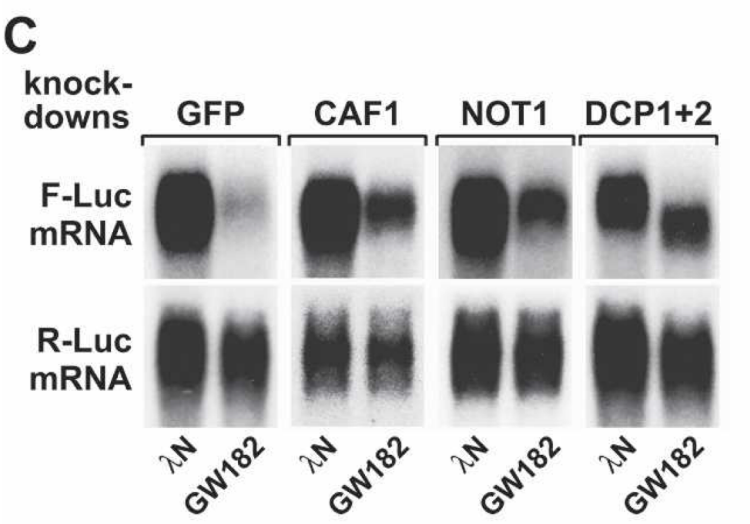


Northern Blots

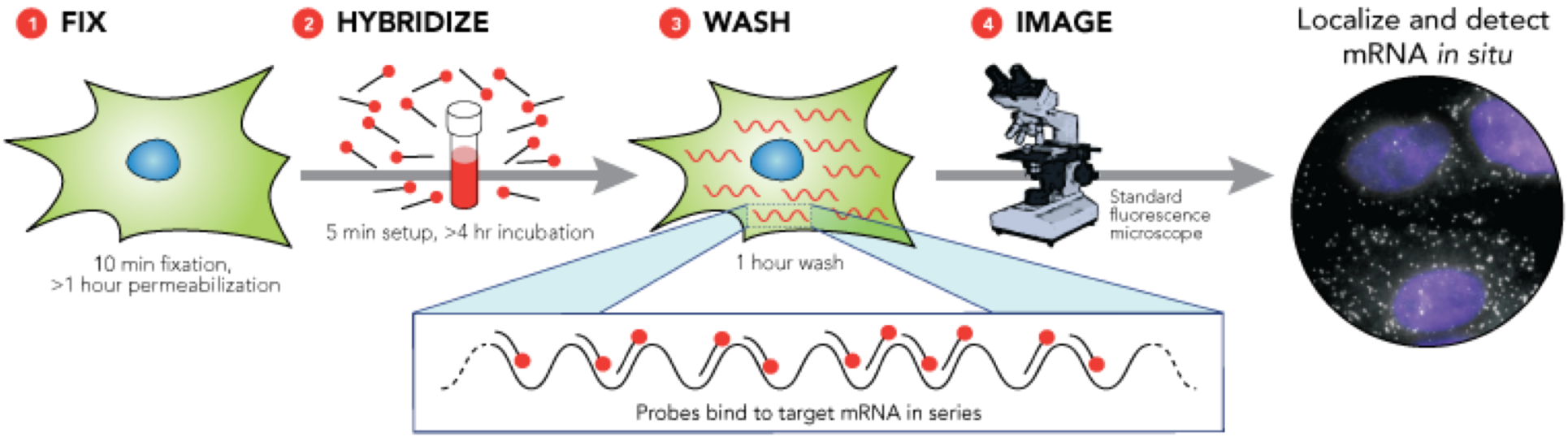


Northern Blots - examples

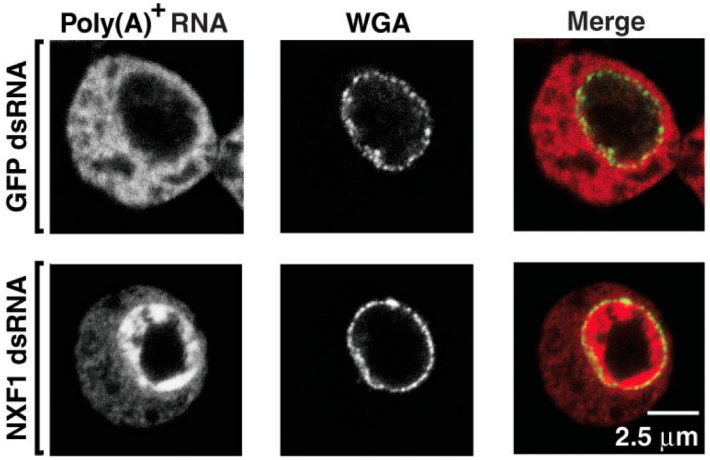




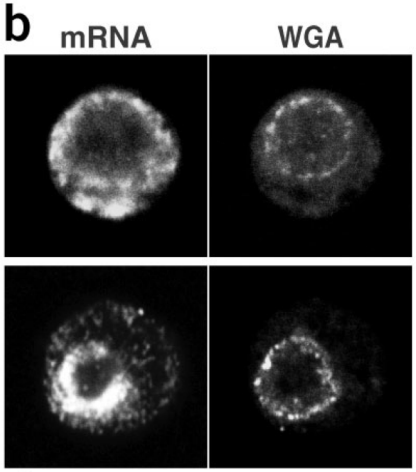
RNA fluorescence in situ hybridisation (FISH)



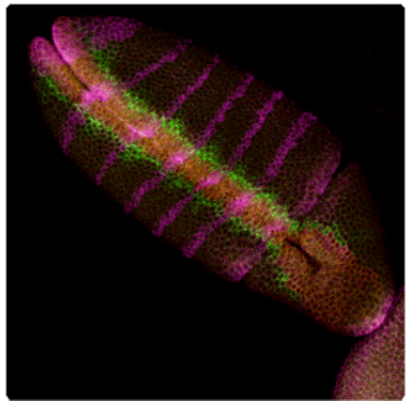
Cy-3-labeled oligo(dT) probe (detects poly(A)-tail of mRNAs)



digoxigenin-labeled specific RNA probe for Hsp70

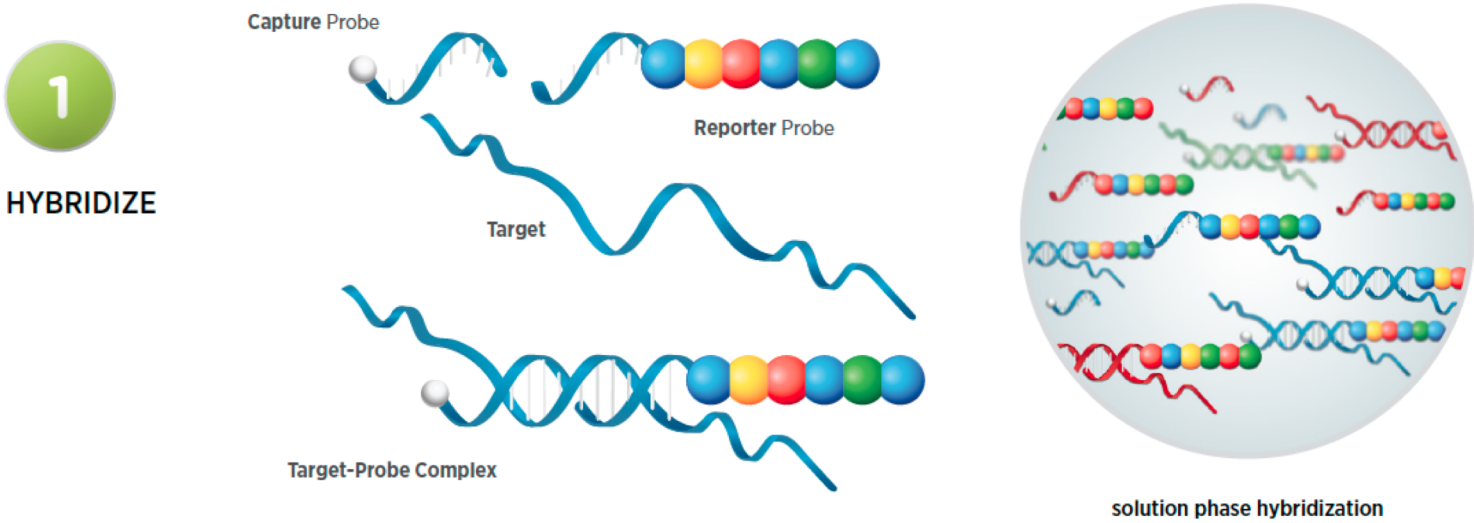


multi-colour FISH

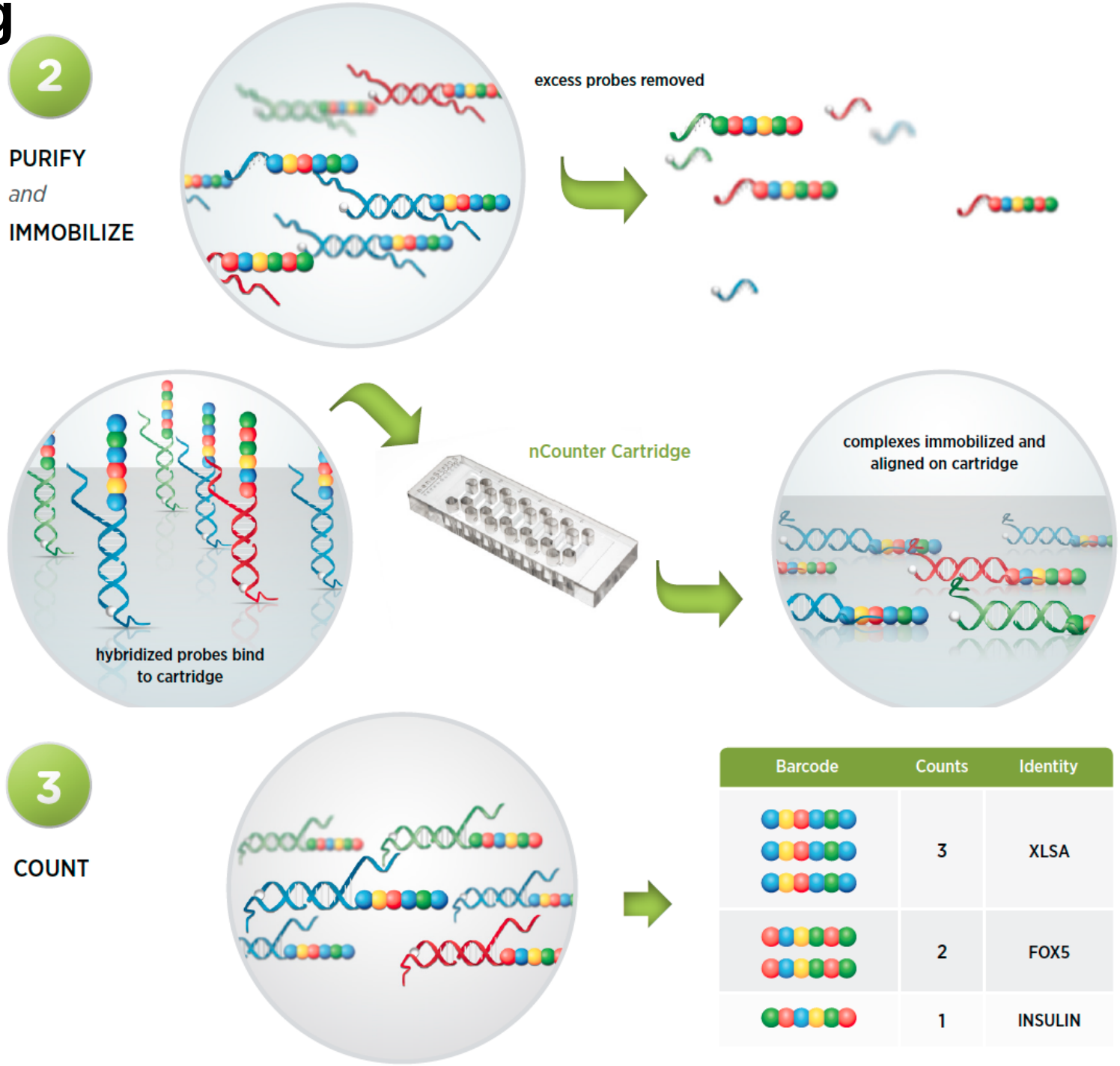


Detecting, quantifying and characterizing pools of different RNAs

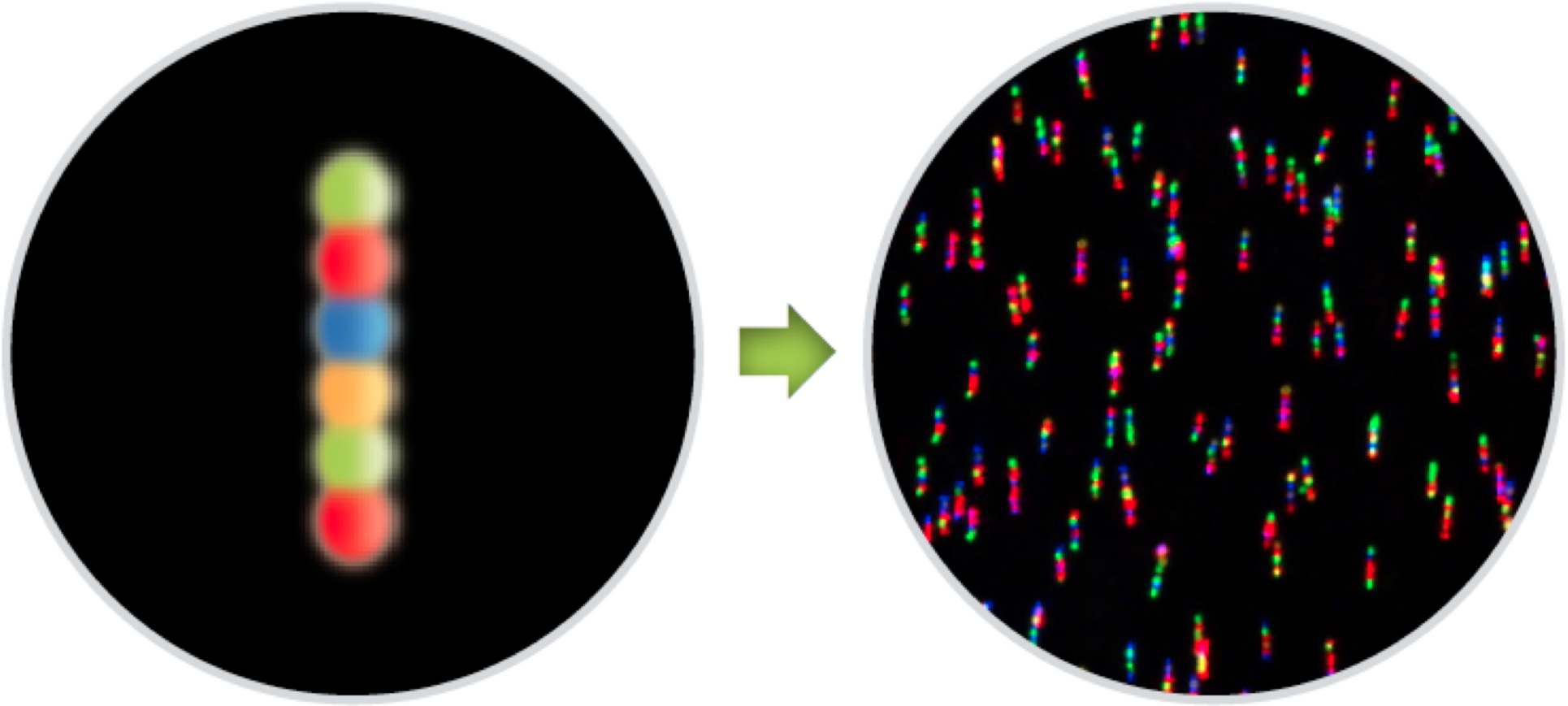
NanoString


1
HYBRIDIZE
Capture Probe
Reporter Probe
Target
Target-Probe Complex
solution phase hybridization

NanoString


2
PURIFY
and
IMMOBILIZE
excess probes removed
hybridized probes bind
to cartridge
nCounter Cartridge
complexes immobilized and
aligned on cartridge
3
COUNT
Barcode
Counts
Identity
3
XLSA
2
FOX5
1
INSULIN

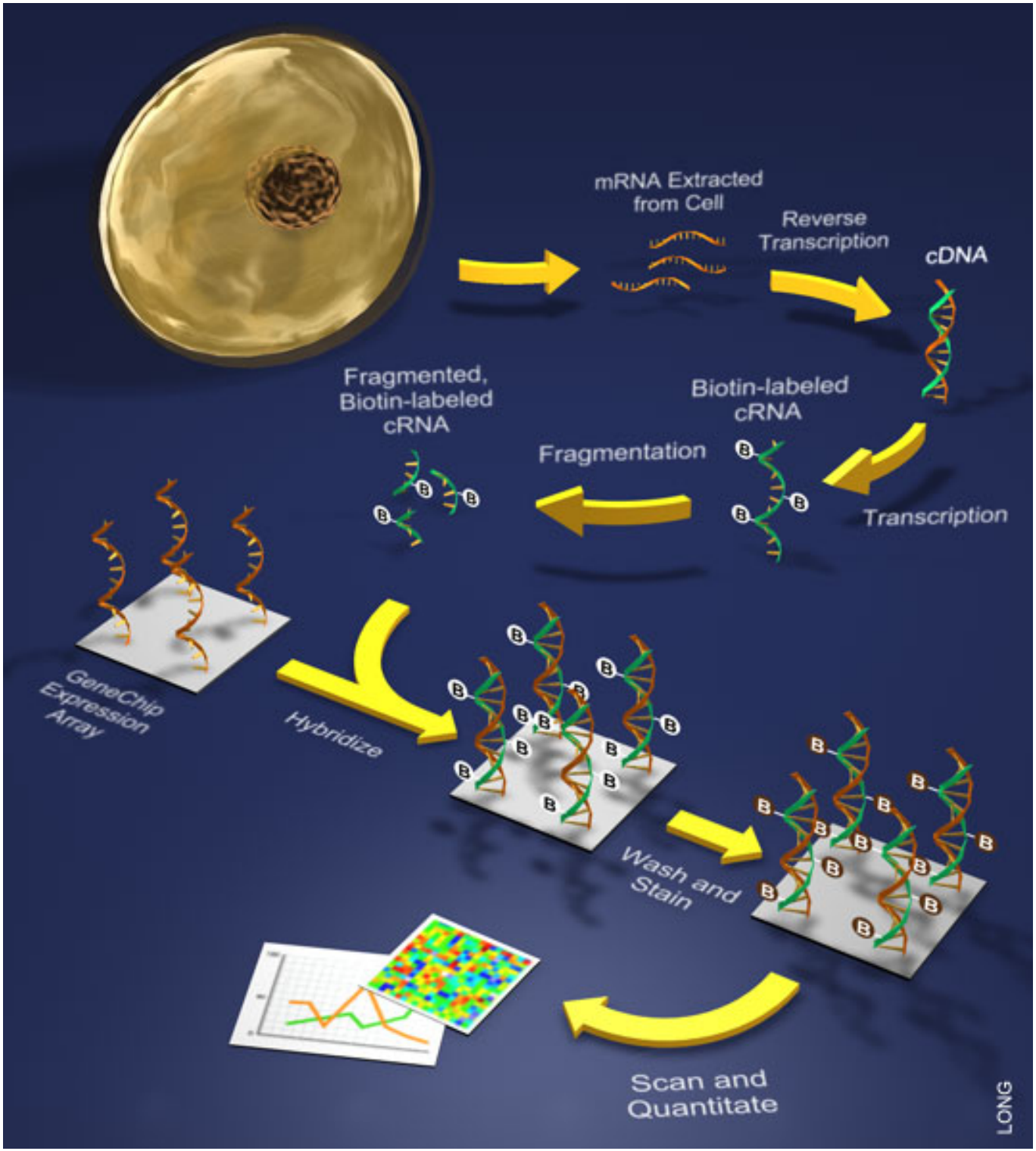
NanoString

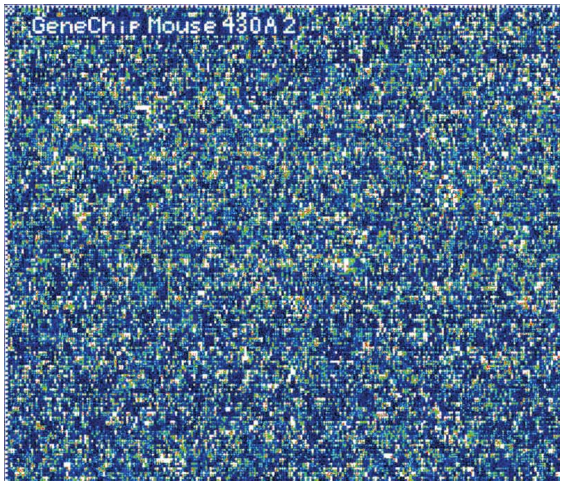


- several hundred transcripts analyzed in parallel
- no amplification

Microarrays

in-situ synthesized oligonucleotide arrays





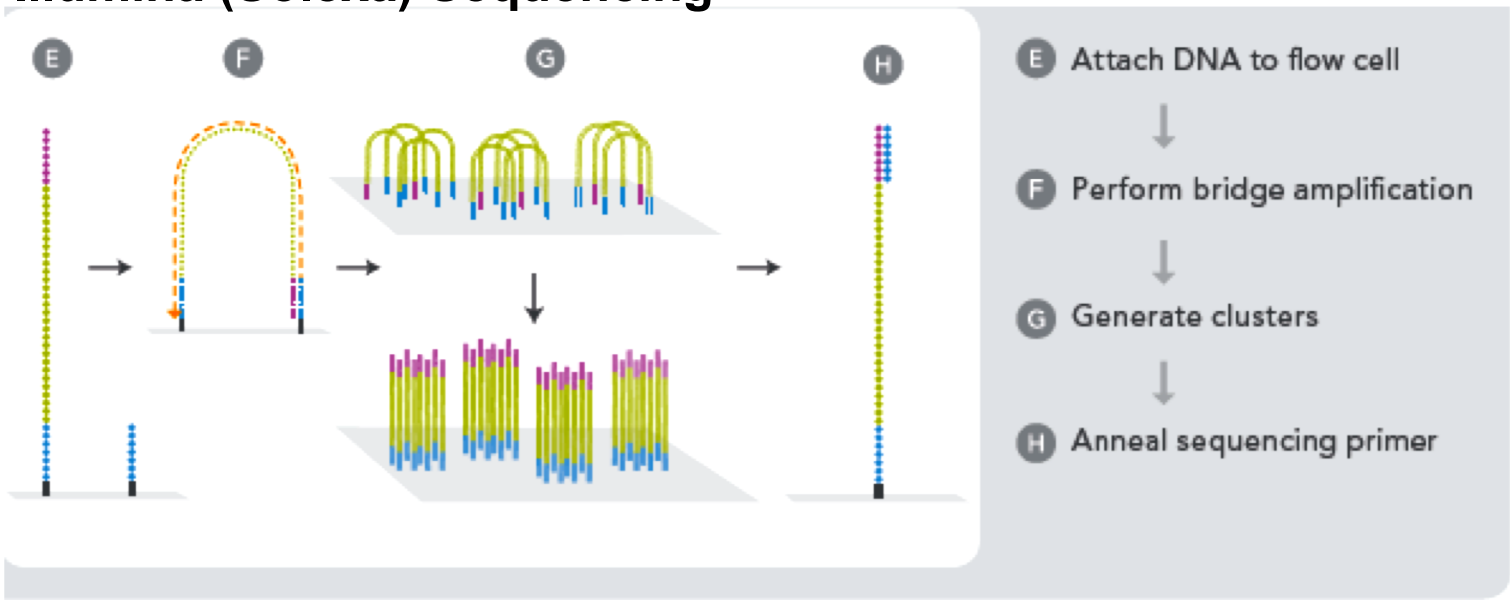
RNA sequencing

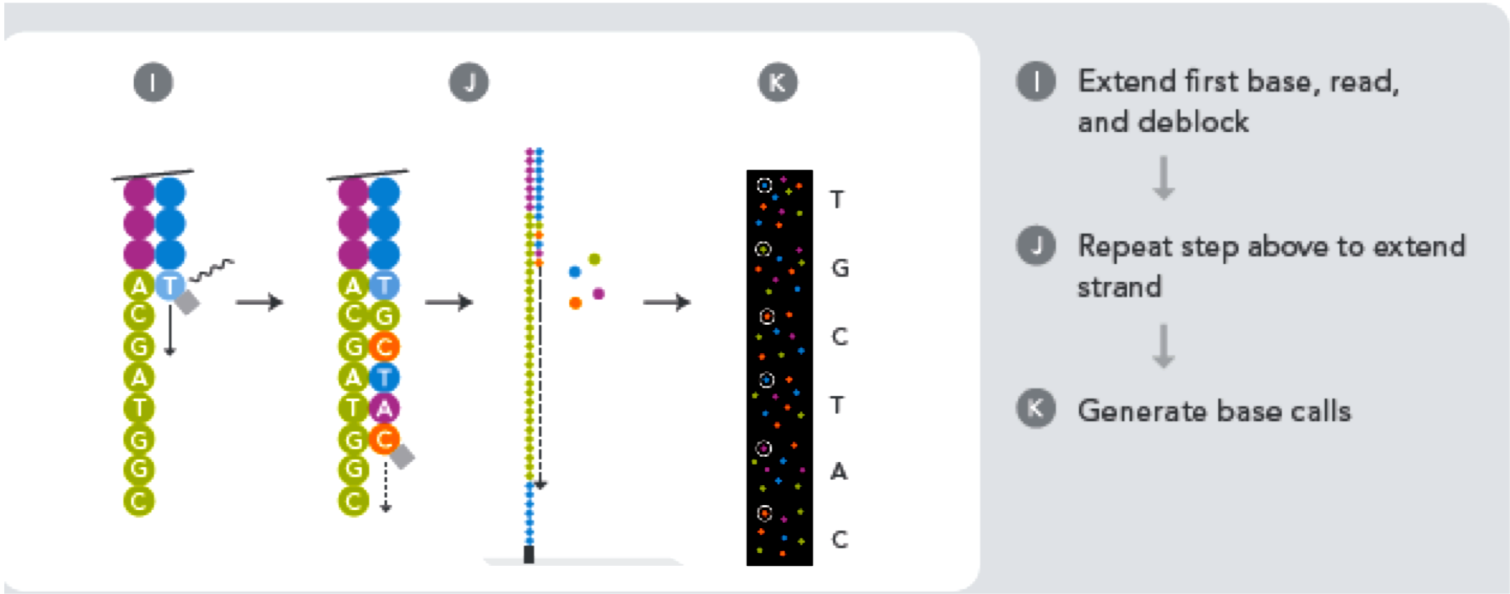
preparation of library

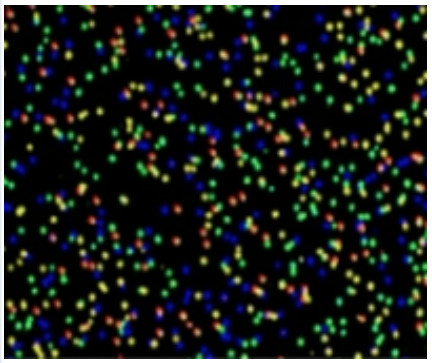
1. Purify RNA
2. Bind polyA fraction (mRNA)
3. Fragment RNA (200 bp)
4. Convert to cDNA by random priming
5. Apply adaptors and sequence

RNA sequencing

Illumina (Solexa) Sequencing





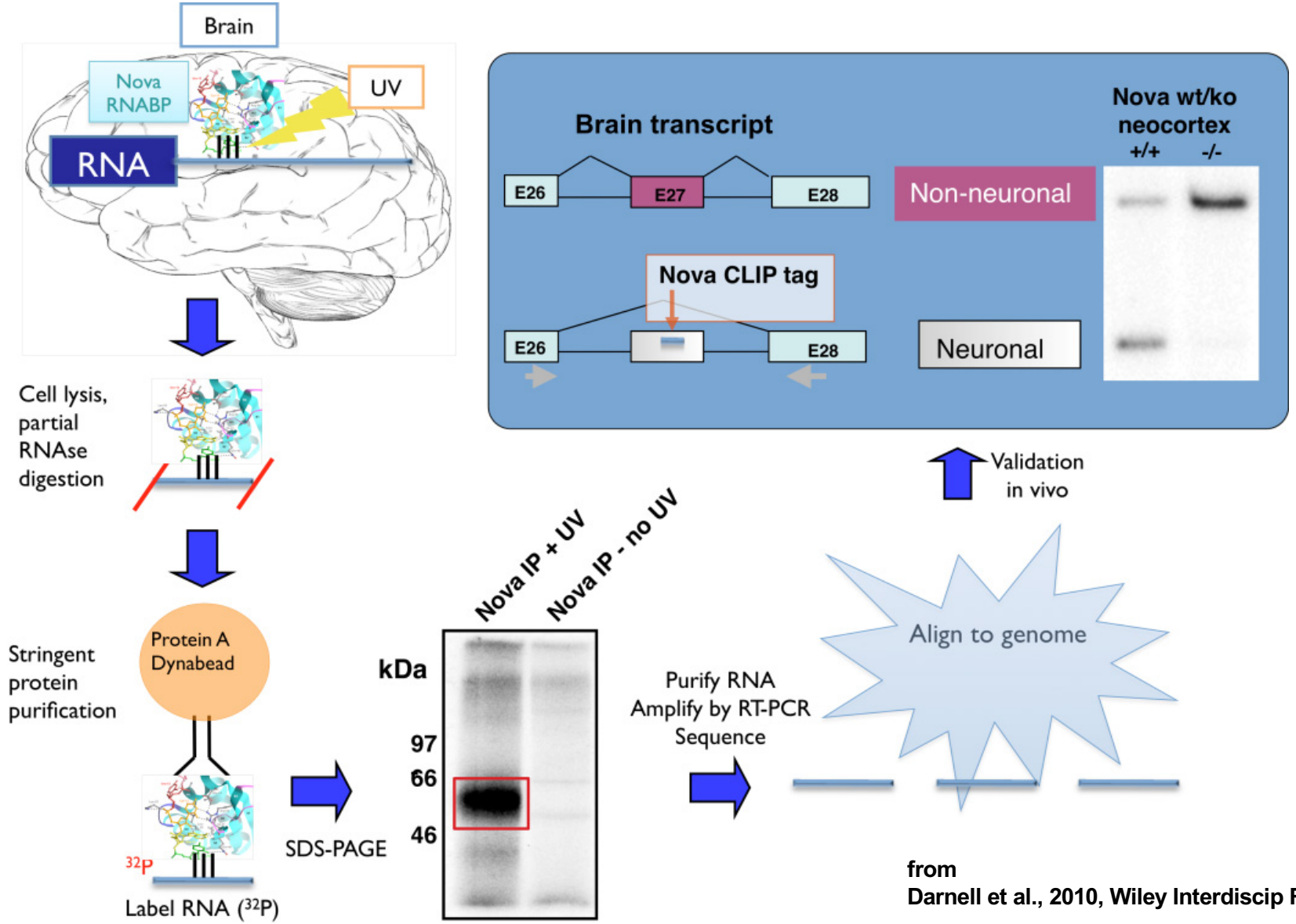


Studying RNA-protein interactions

1. Identification of RNAs that bind a known protein
2. Identification of proteins that interact with a given RNA

Identification of RNAs that bind a known protein

CLIP (cross-link immunoprecipitation)

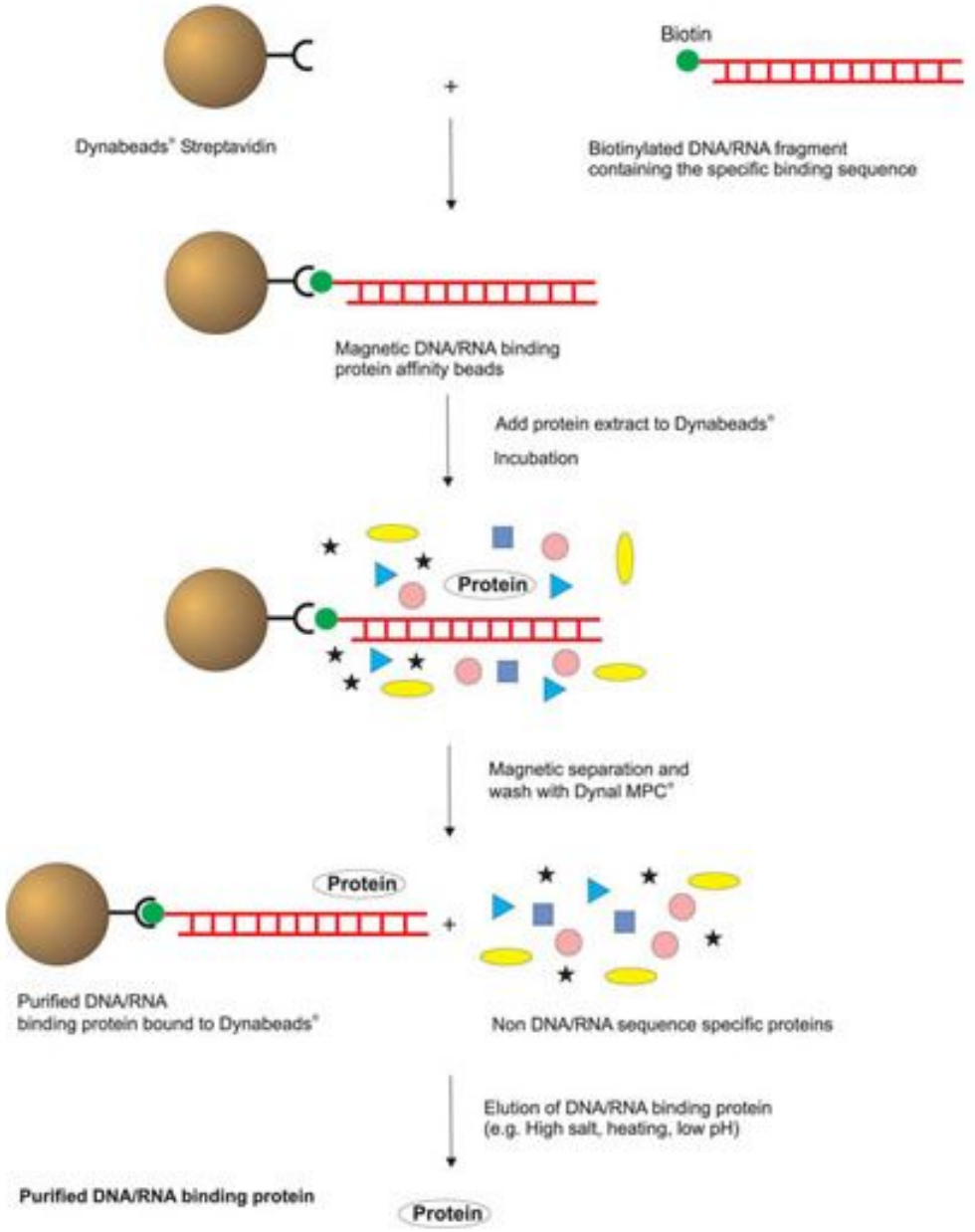


from
Darnell et al., 2010, Wiley Interdiscip Rev RNA.

Identification of proteins interacting with a given RNA

1) **pull-downs with *in-vitro* transcribed, biotinylated RNA**

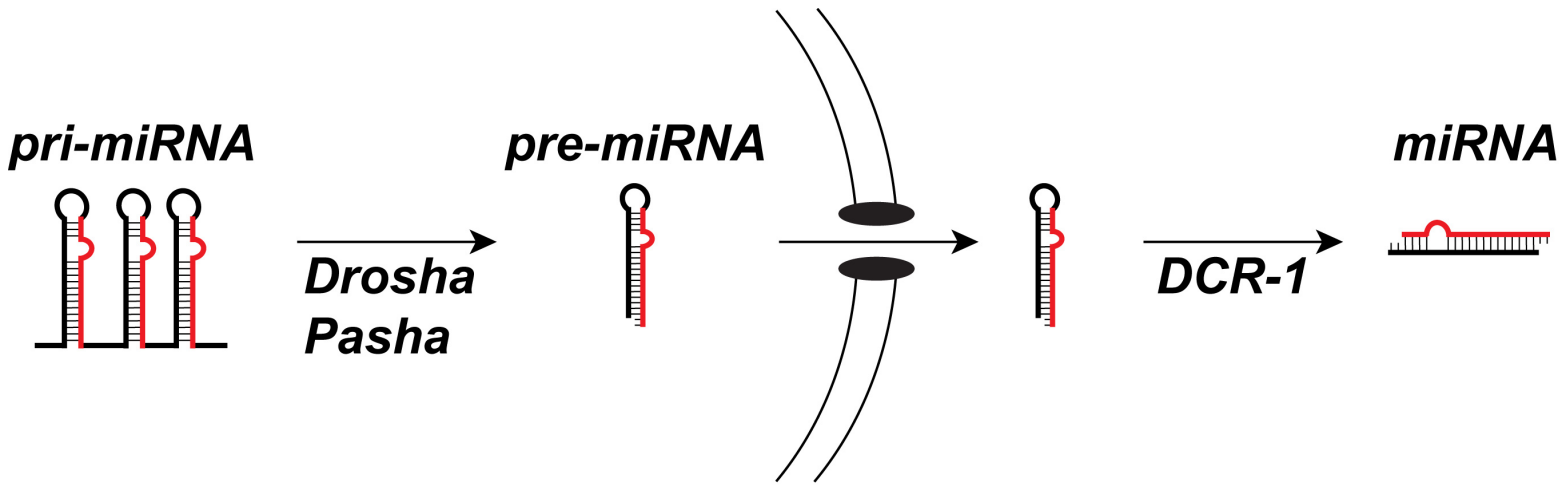
   ➔ RNA and protein associate in the test tube



RNA interference

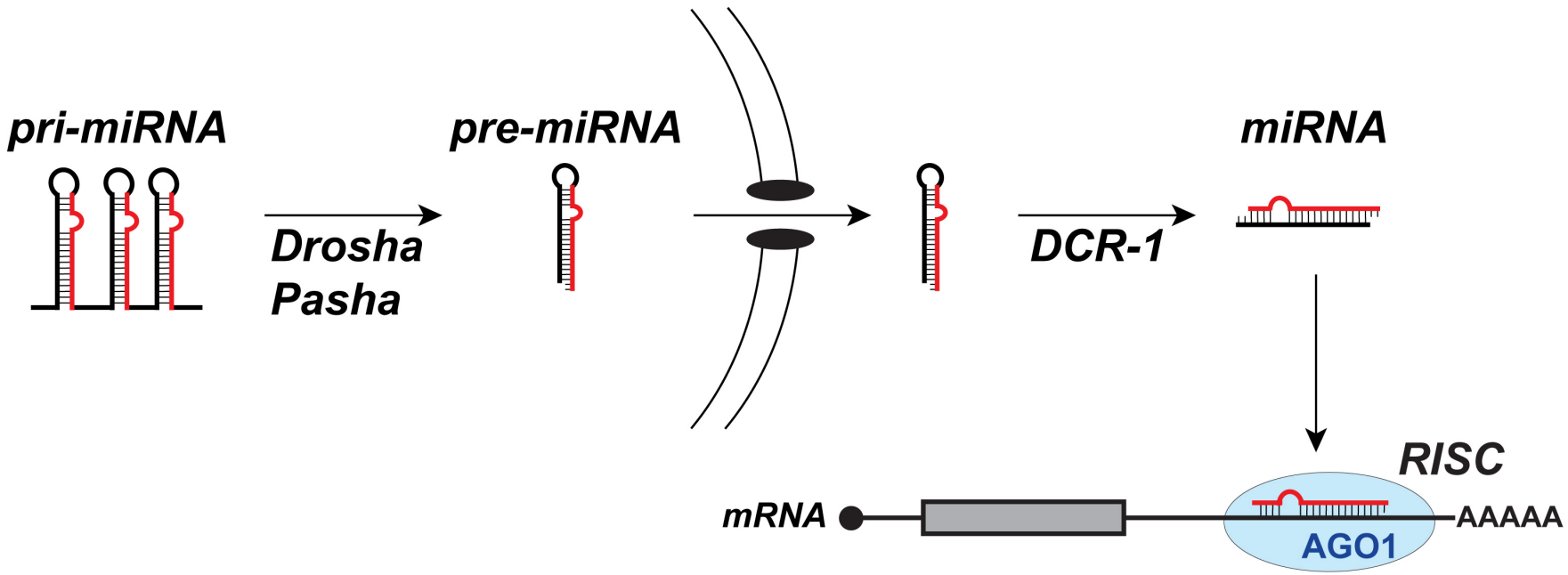
“knock-downs”

miRNA-mediated gene silencing



Bartel, 2004, *Cell* 116:281; Filipowicz, 2005, *Cell* 122:17; Wienholds & Plasterk, 2005, *FEBS Letters* 579:5911; Jackson & Standart, 2007, *Science* STKE:367; Nilsen, 2007, *Trends Genet.* 23:243

miRNA-mediated gene silencing



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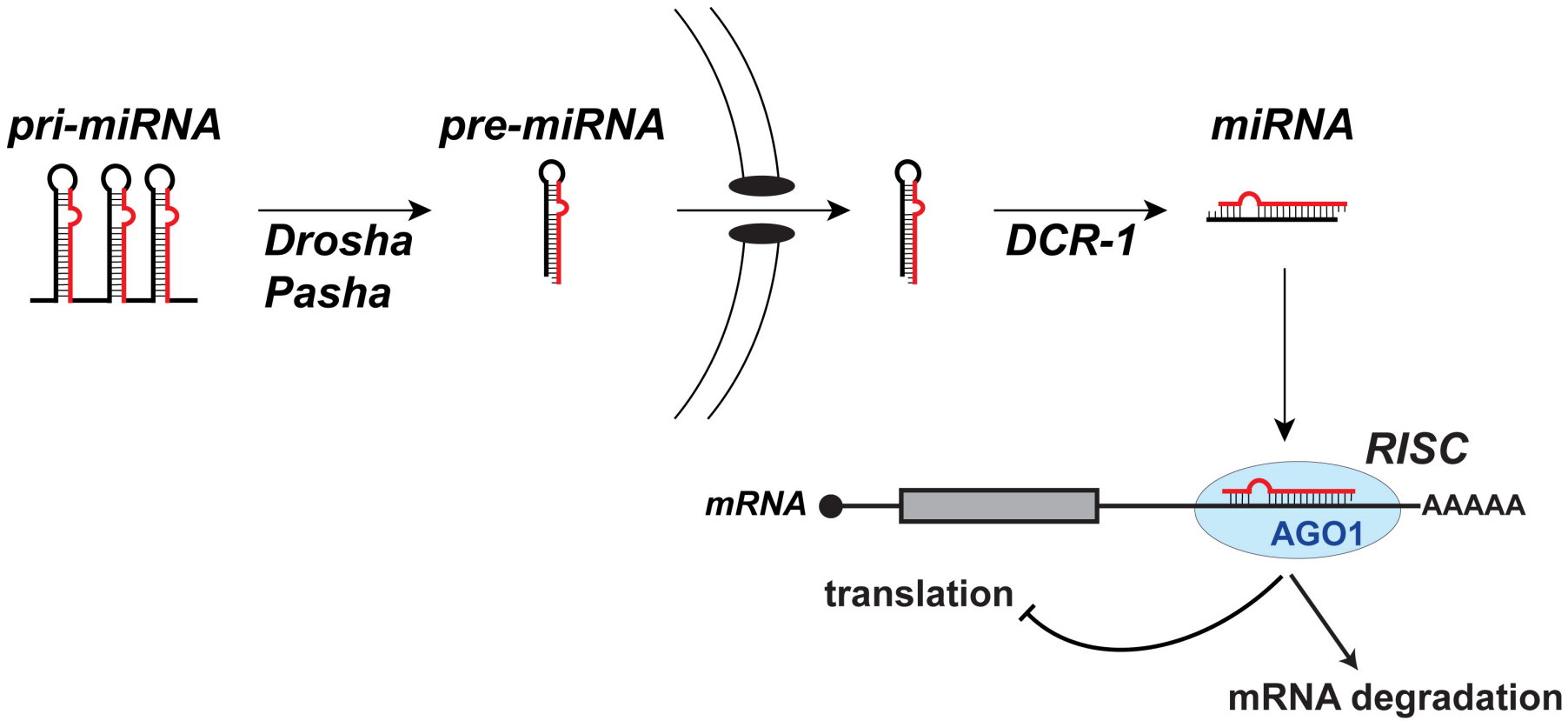
miRNA-mediated gene silencing



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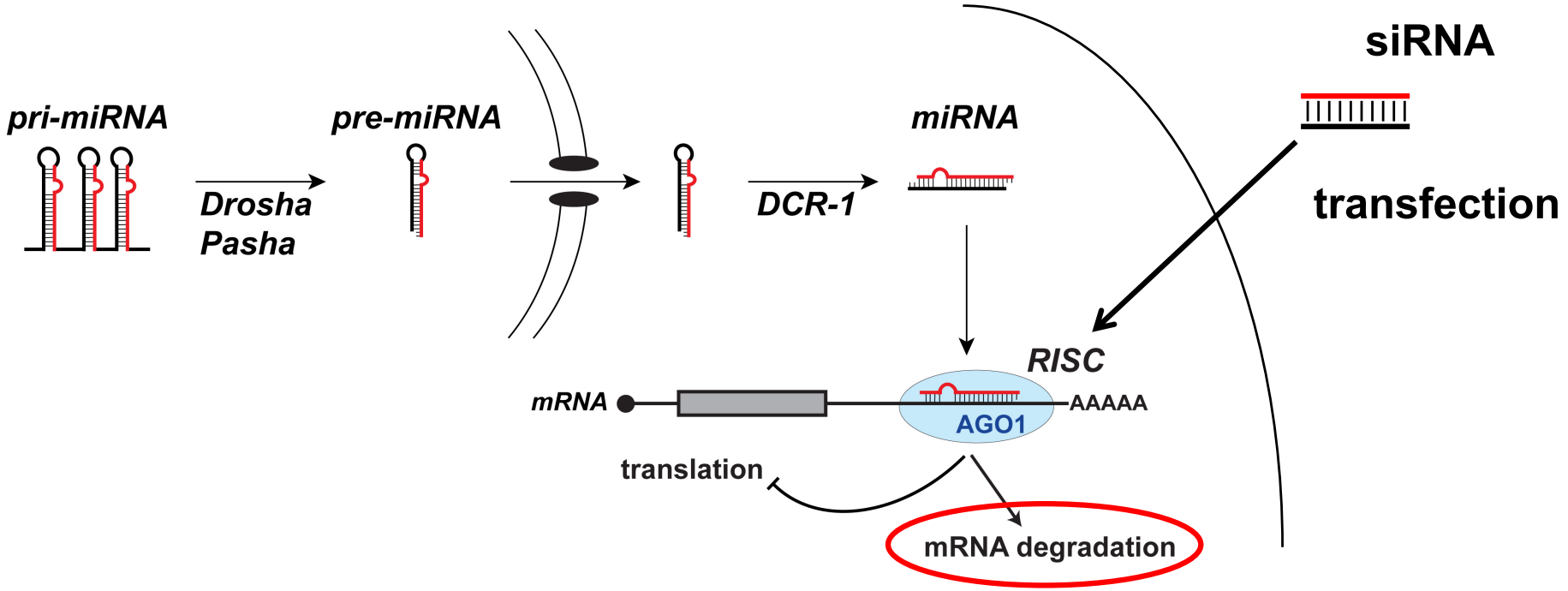
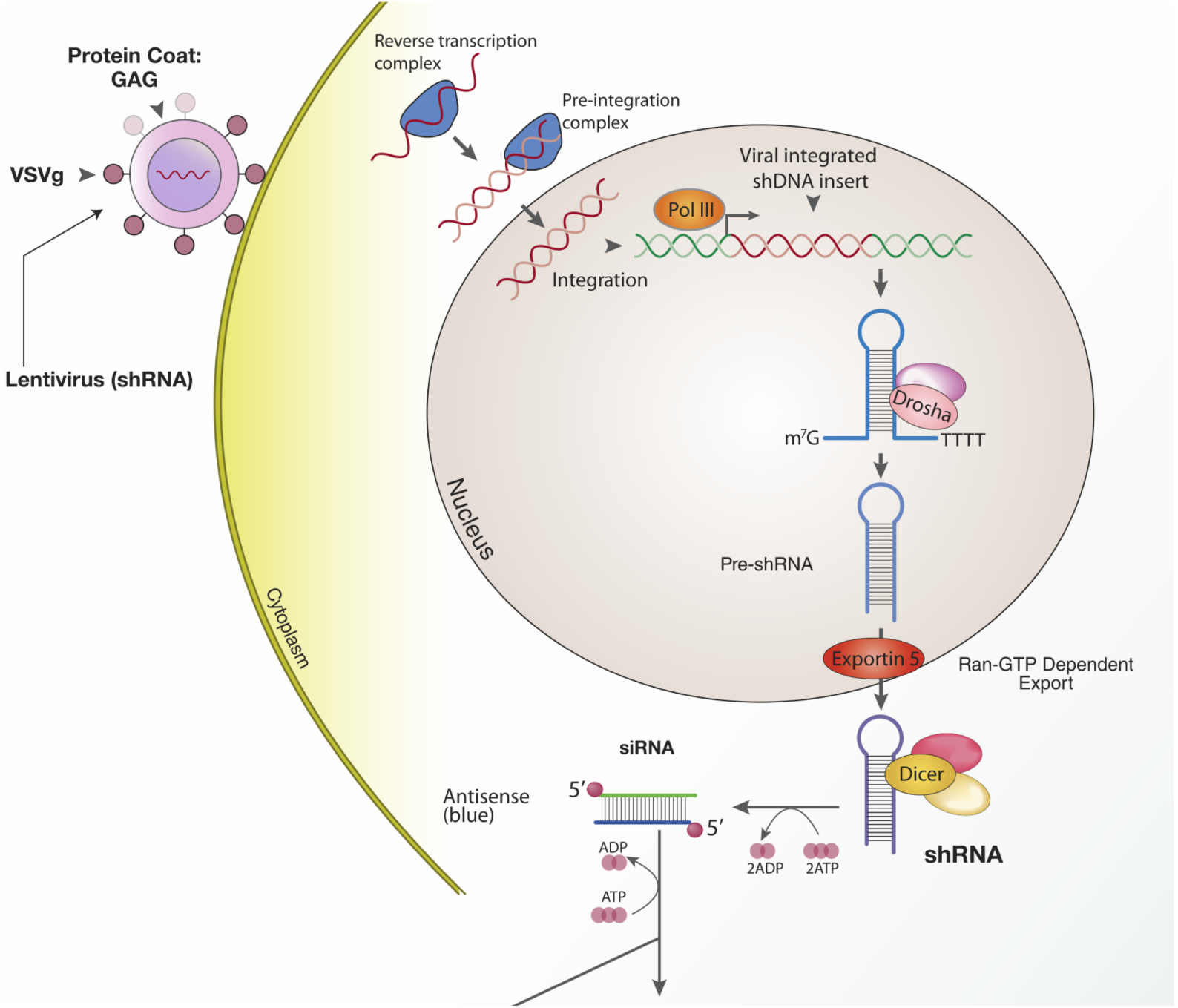
siRNAs

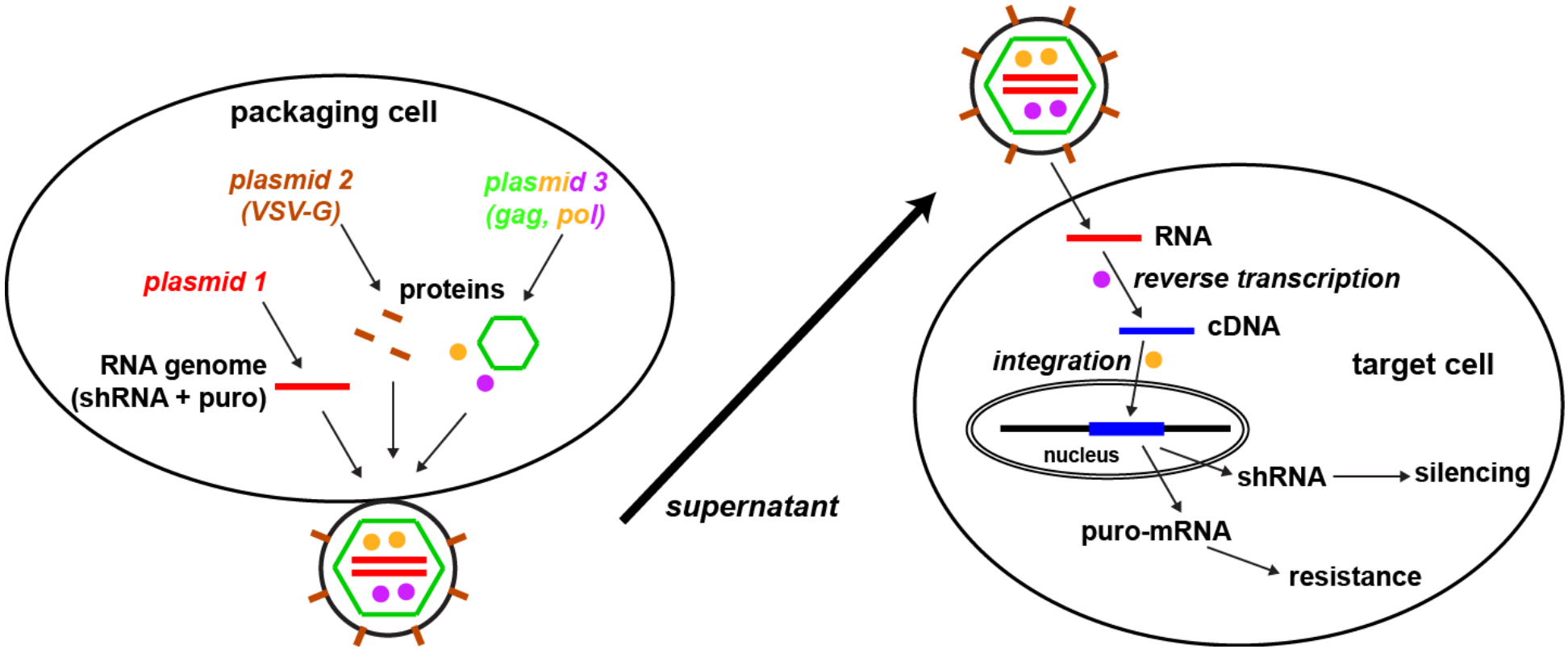


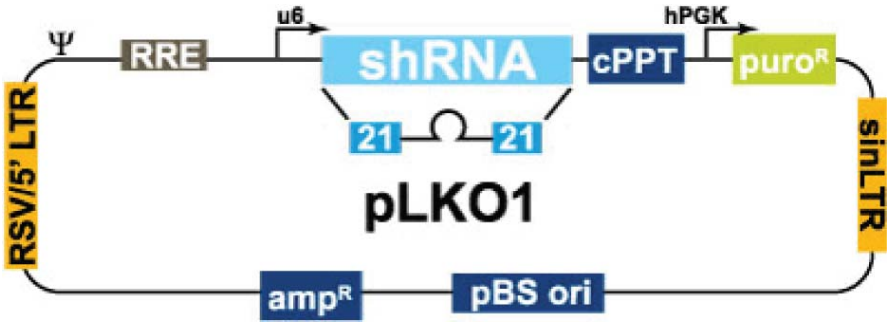
- validate knock-down efficiency by Western blot or qRT-PCR ➔ many siRNAs work poorly or not at all
- always use at least two different siRNAs ➔ off-target effects!
- “smartpools” – mix of four different siRNAs to reduce off-target effects; needs validation using a different “pool” or by testing siRNAs separately
- results may be difficult to interpret if the knockdown impacts on cell viability
- if possible, also use two control siRNAs

shRNAs

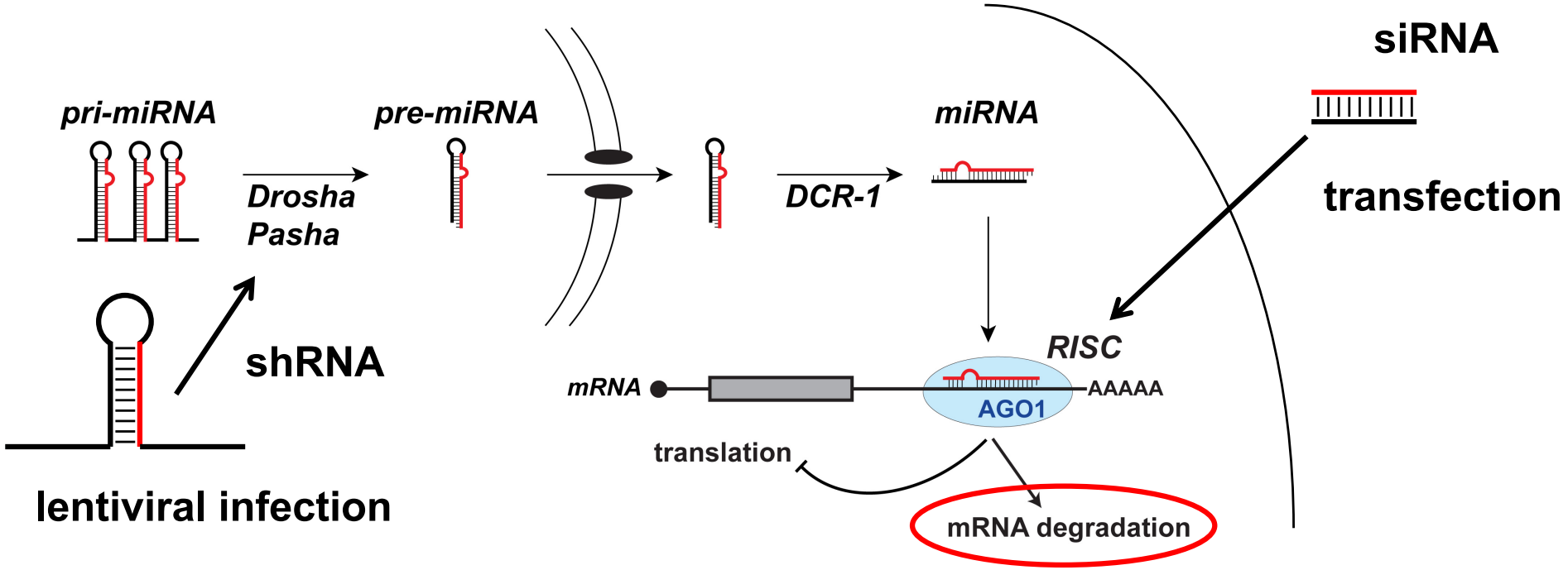

Protein Coat:
GAG
VSVg
Lentivirus (shRNA)
Reverse transcription
complex
Pre-integration
complex
Integration
Viral integrated
shDNA insert
Pol III
Drosha
m7G
TTTT
Nucleus
Cytoplasm
Pre-shRNA
Exportin 5
Ran-GTP Dependent
Export
Dicer
shRNA
siRNA
Antisense
(blue)
5′
5′
ADP
ATP
2ADP
2ATP
RISC

lentiviral vectors





shRNAs vs. siRNAs



	Delivery	Notes
siRNAs	transfection	• transient knockdown • fast & easy to use • depletion efficiency may vary from cell to cell • not all cells are easy to transfect
shRNAs	lentiviral infection	• stable knockdown; cell lines can be selected • suitable to many different types of cells • labour intensive; lentiviruses = BSL cat 2!