



# ABRF 2018 Annual Meeting

*Genomics Track*

## Research Group & Platinum Level Vendor Presentations

- Stuart Levine, Massachusetts Institute of Technology
- Justin J. Lemke, Promega Life Sciences
- John M. Ashton, University of Rochester
- **Break**
- Steve Siembieda, Advanced Analytical Technologies, Inc
- Christopher E. Mason, Weill Cornell Medicine
- Nancy Nabilisi, Roche



*Thank you for sponsoring beverages!*

Myrtle Beach Convention Center - Myrtle Beach, South Carolina

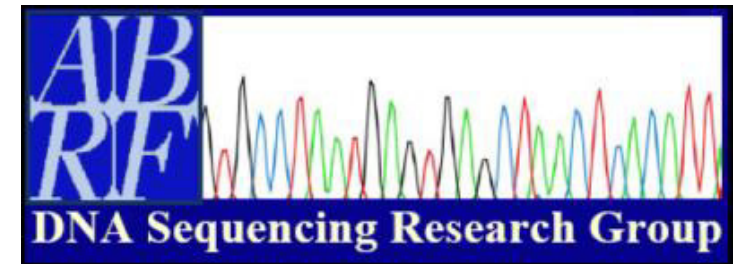


# ABRF 2018 Annual Meeting

A MEETING OF THE ASSOCIATION OF BIOMOLECULAR RESOURCE FACILITIES

## DSRG 2017

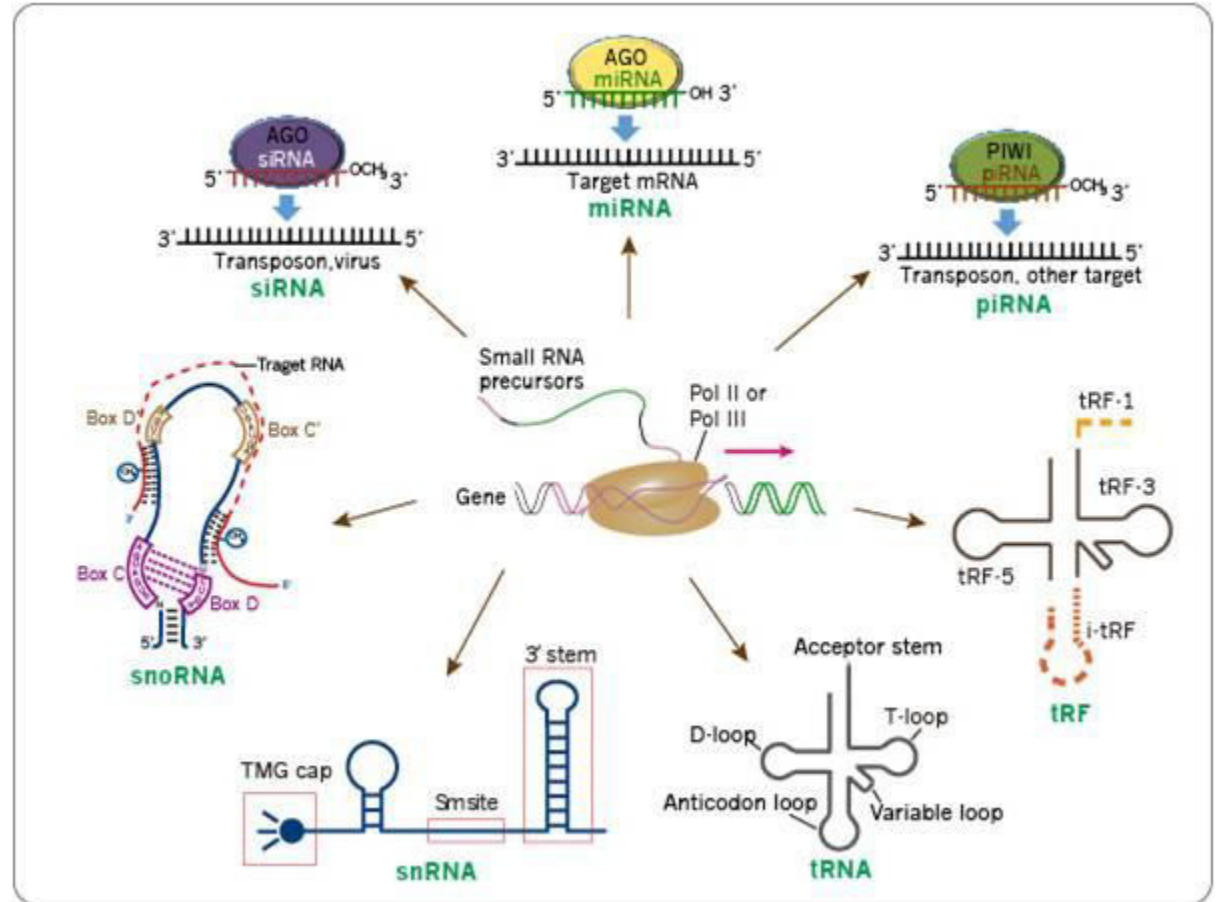
### Comparison of small RNA chemistries



April 22-25, 2018

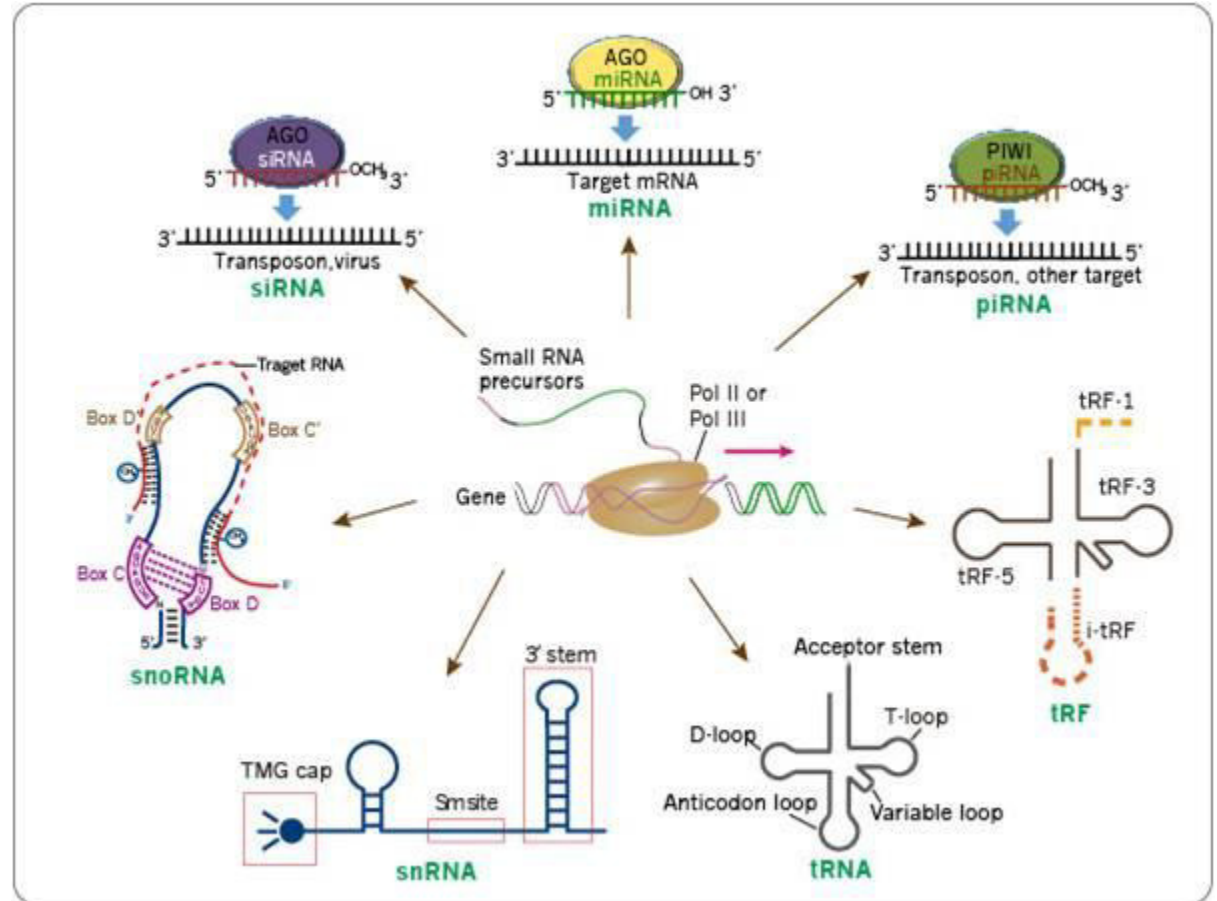
Myrtle Beach Convention Center - Myrtle Beach, SC

# Small RNA



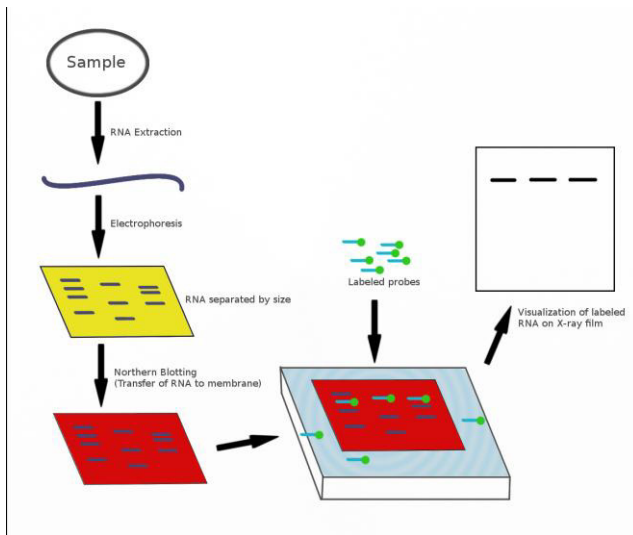
# Small RNA

- Generally <100nt
- Often lost from standard RNA isolation methods
- Too short for random priming
- Not detected reproducibly in standard RNAseq



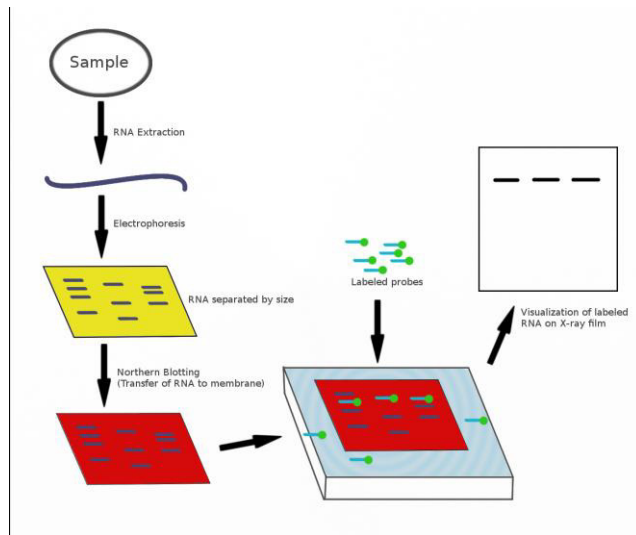
# Small RNA Detection

## NORTHERN BLOT

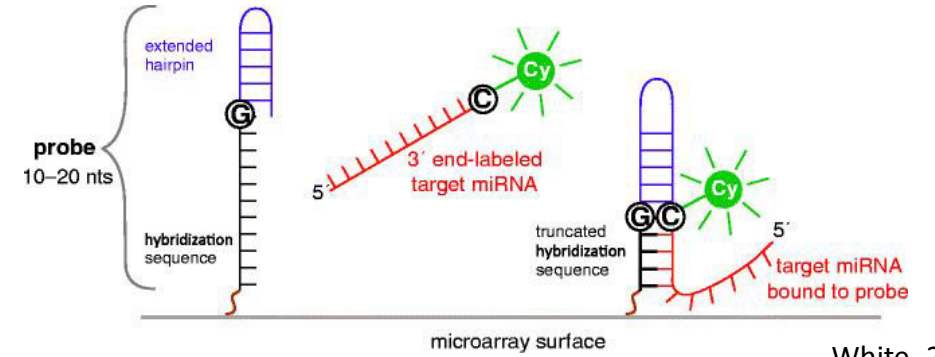


# Small RNA Detection

## NORTHERN BLOT

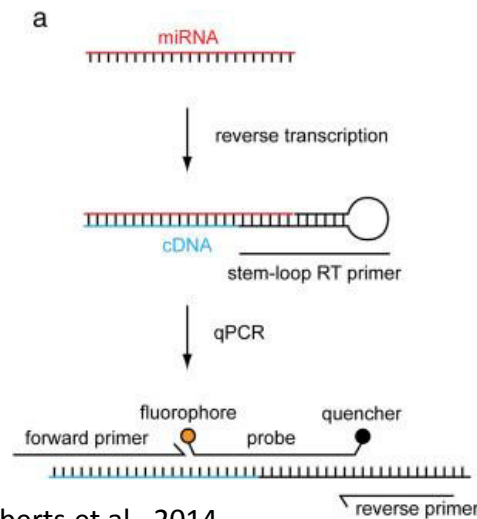


## MICROARRAY



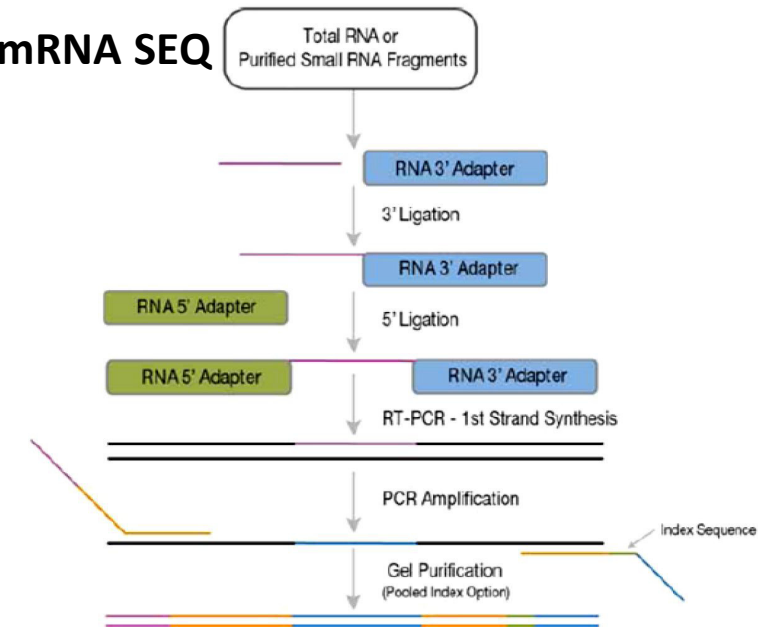
White, 2011

## qPCR

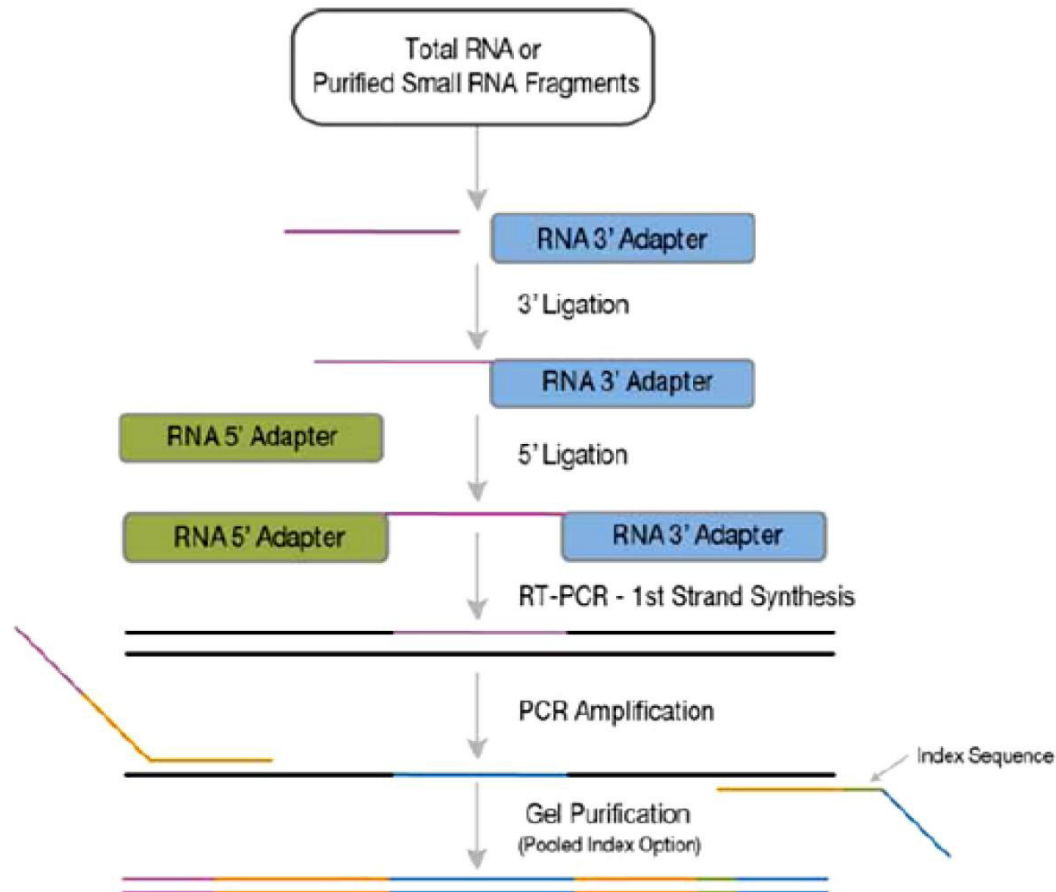


Roberts et al., 2014

## smRNA SEQ



# Small RNA Applications



- **miRNA Detection**
- **other small RNAs (eg PIWI)**
- **tRNA regulation**
- **CLIP-SEQ**
- **Ribosomal Footprinting**
- **Sequential ligation for mRNA**

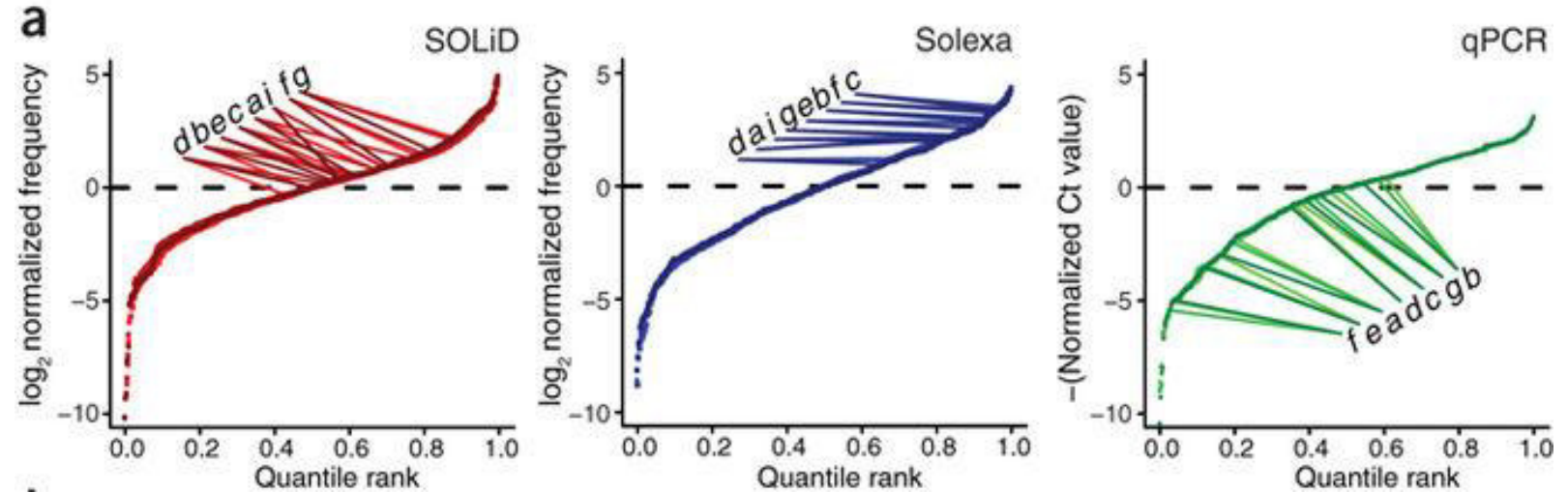
# BIAS IN SMALL RNA DETECTION

- GIVEN EQUIMOLAR SAMPLE  
All methods showed highly non-linear behavior

Bias was KIT SPECIFIC such that no absolute value could be correctly interpreted.

Bias is reproducible – running the same kit twice gives the same result

\* RELATIVE values are robust, ABSOLUTE values are not



Linsen et al., Nature Methods 2009



# NEW CHEMISTRIES

illumina®

 **BIO SCIENTIFIC®**  
a PerkinElmer company

nanoString™  


 *NEW ENGLAND*  
**BioLabs®**

 **TakaRa**  
Clontech TakaRa cellartis

somagenics

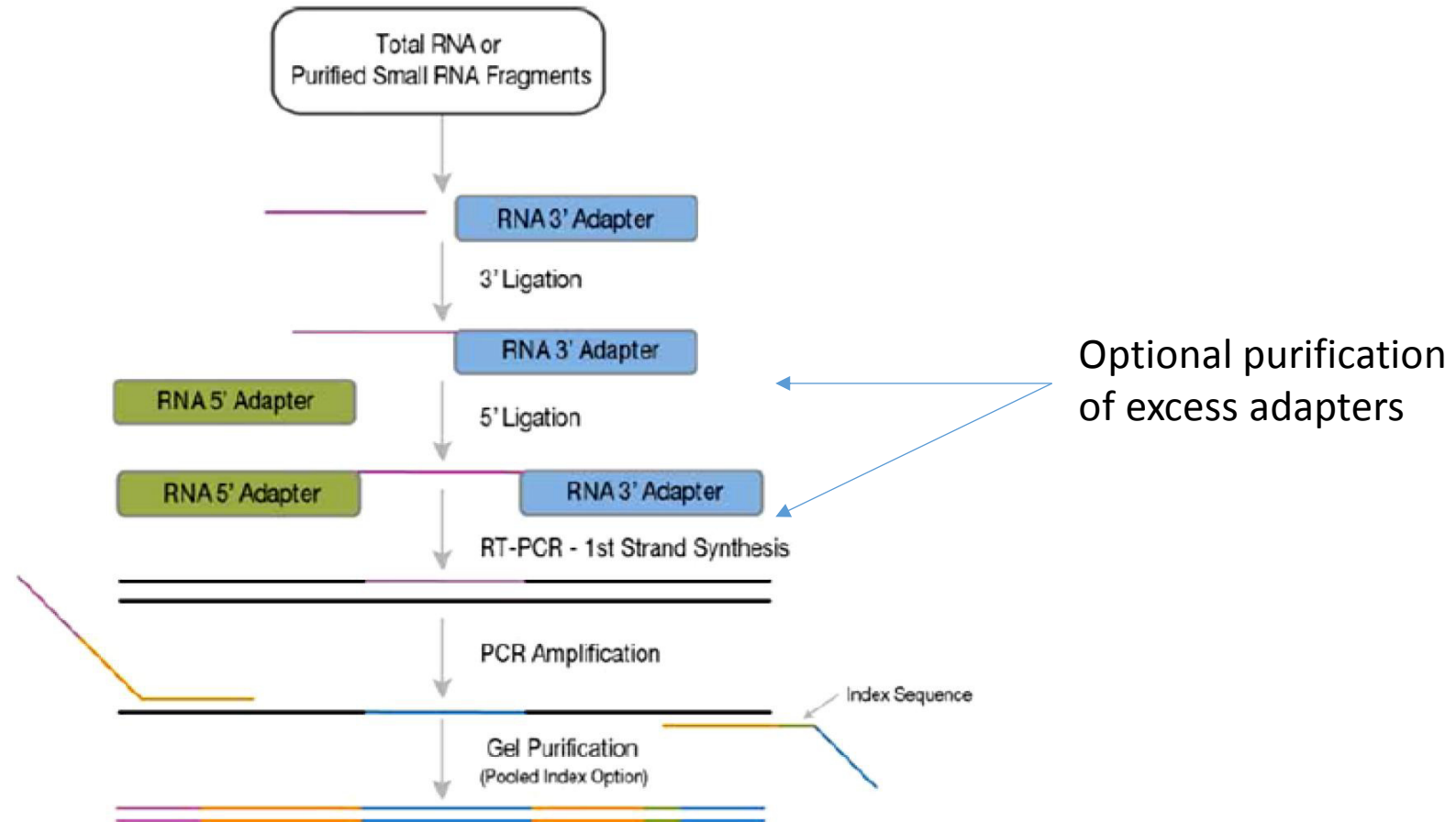
  
**QIAGEN**

diagenode

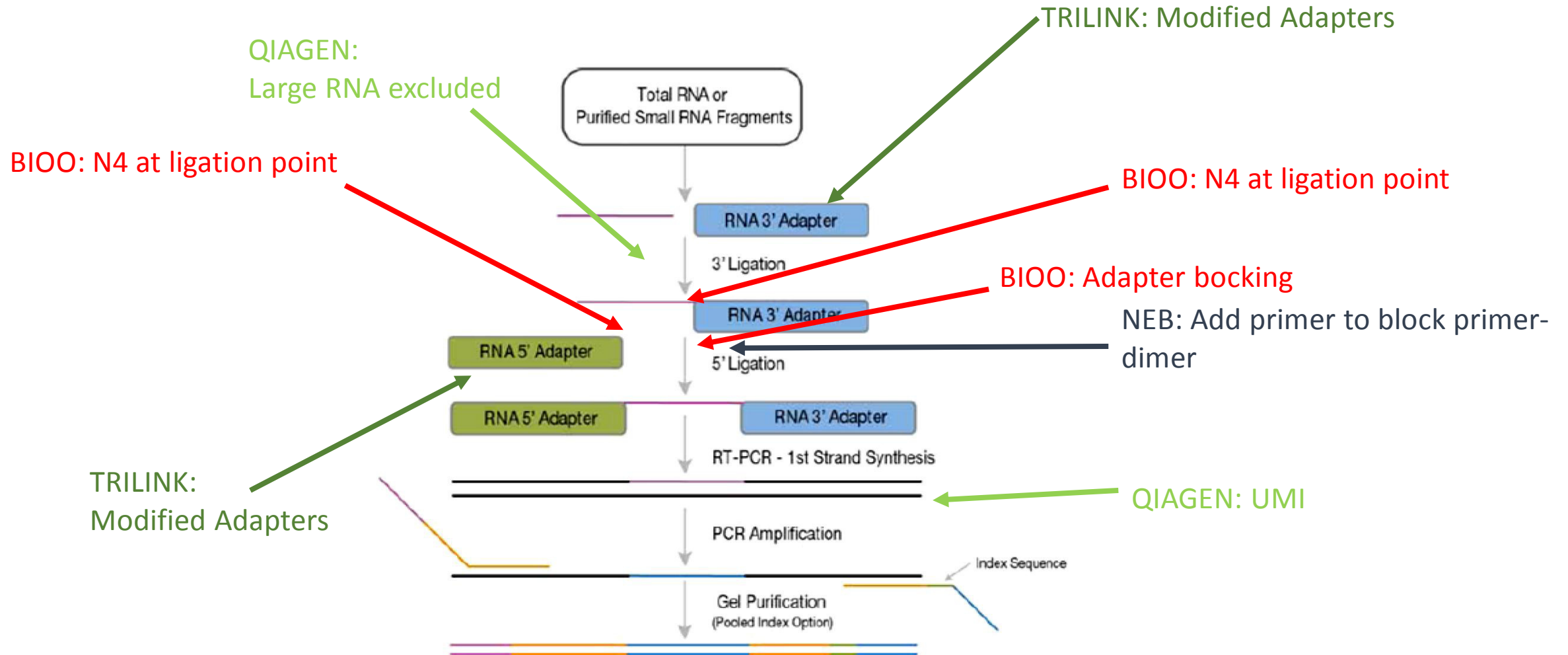
 **TriLink®**  
BIOTECHNOLOGIES  
part of Maravai LifeSciences

**LEXOGEN**  
Enabling complete transcriptome sequencing

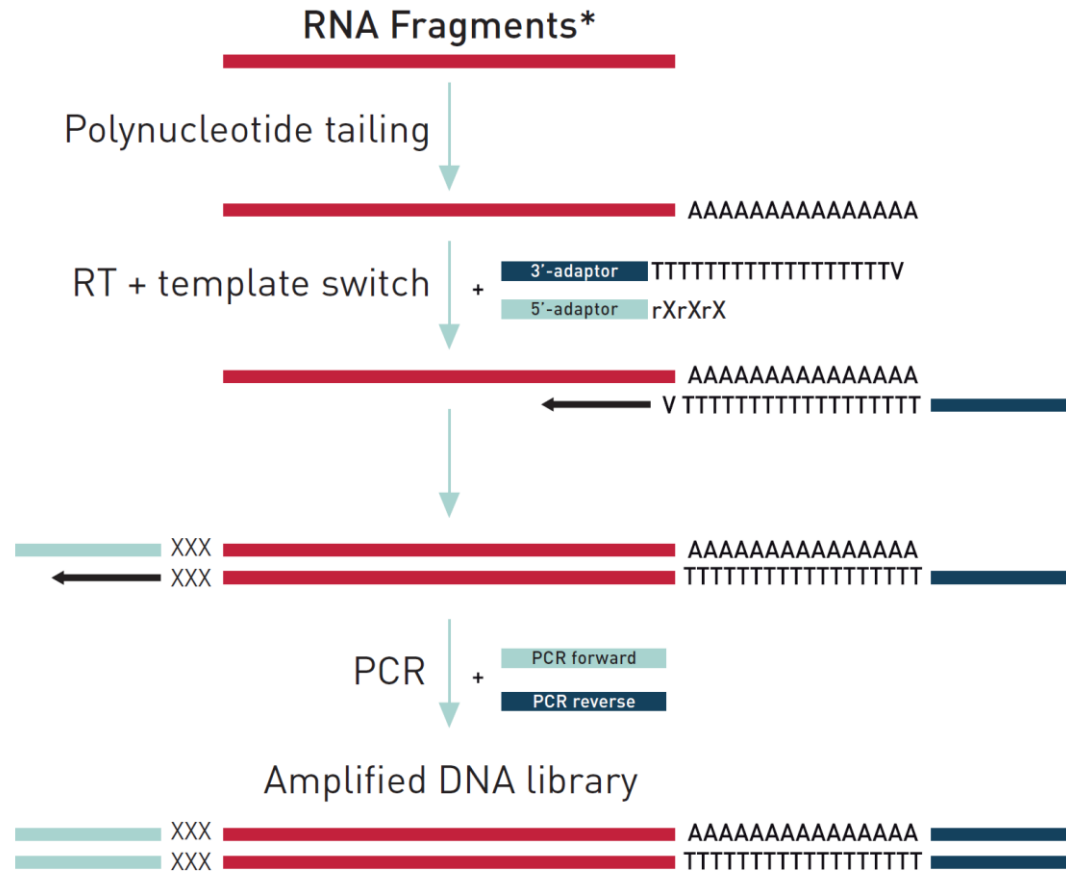
# Sequential Ligation: Illumina, Lexogen



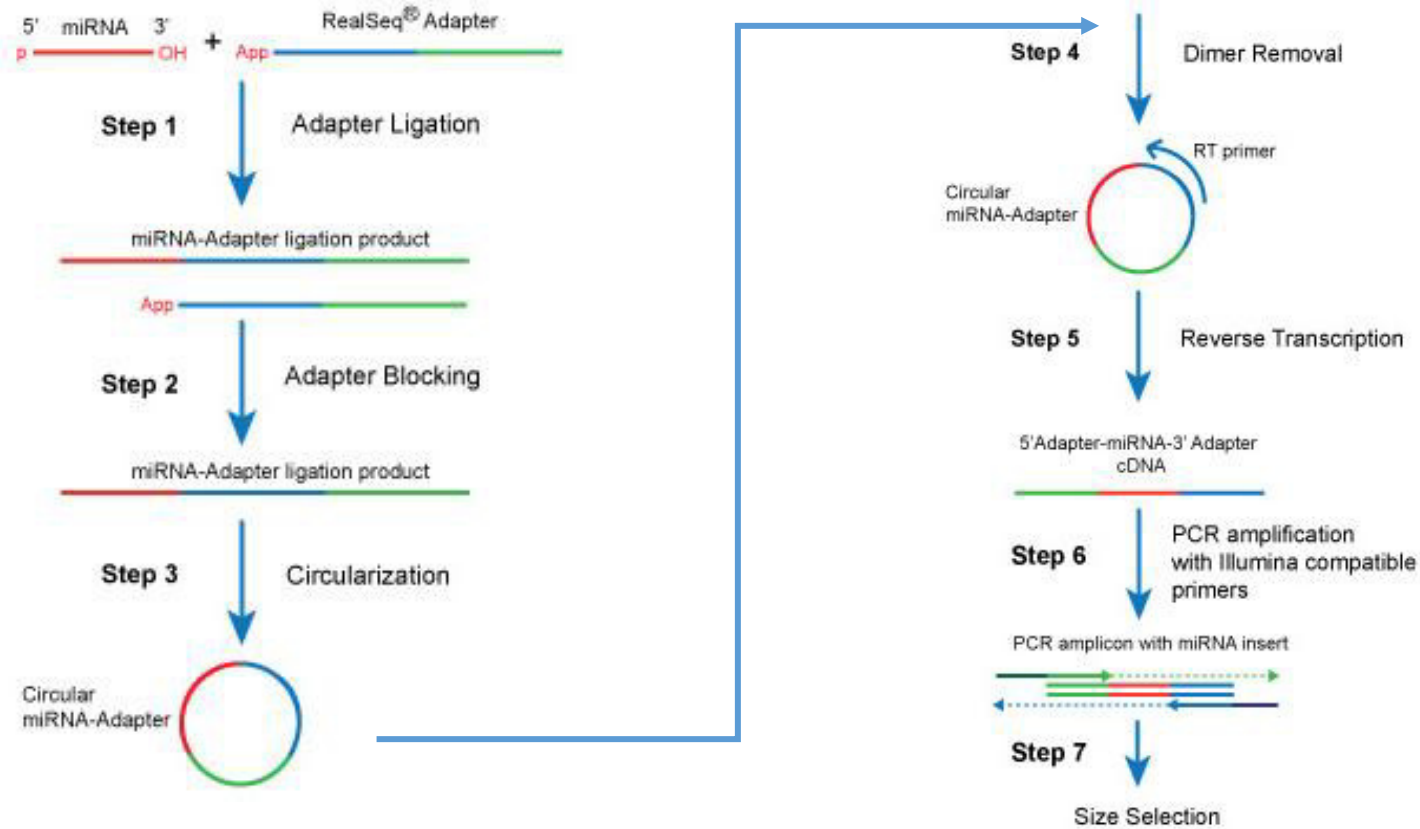
# Sequential Ligation:



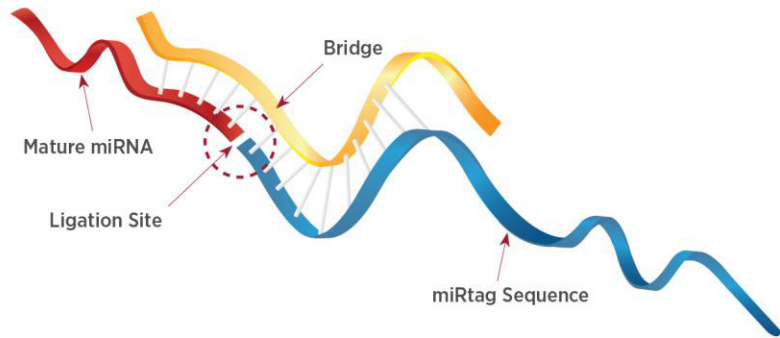
# Template Switching: Clontech(Takara), Diagenode



# Circularization: Rubicon(Takara), Somagenics



# Ligation + Array Hybridization: Nanostring



Step 0: miRtag sequence added to mature miRNA to add length and increase specificity in downstream rxns

**1 Hybridization**

NanoString's Technology employs two ~50 base probes per mRNA that hybridize in solution. The Reporter Probe carries the signal; the Capture Probe allows the complex to be immobilized for data collection.

The diagram shows a 'Target' mRNA strand hybridizing with a 'Capture Probe' (blue) and a 'Reporter Probe' (red). The resulting 'Target-Probe Complex' is shown in a circular inset labeled 'solution phase hybridization'.

**2 Purify and Immobilize**

After hybridization, the excess probes are removed and the probe/target complexes aligned and immobilized in the nCounter Cartridge.

The diagram shows the process of removing 'excess probes' and then 'hybridized probes bind to cartridge'. The final step shows 'complexes immobilized and aligned on cartridge' within an 'nCounter Cartridge'.

**3 Count**

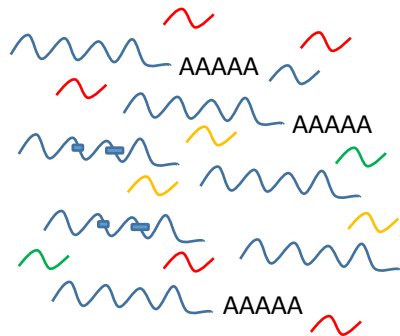
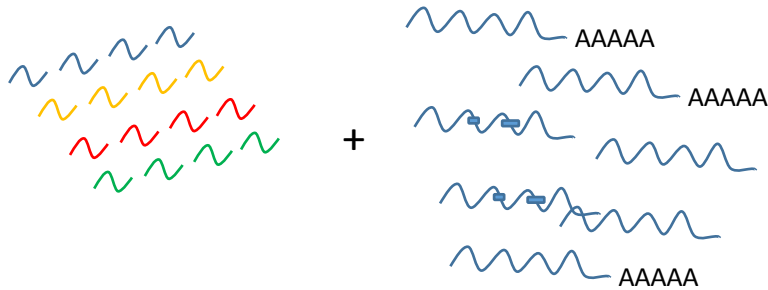
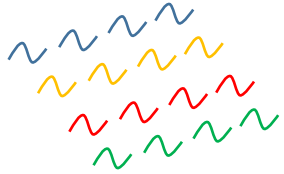
Sample Cartridges are placed in the Digital Analyzer for data collection. Color codes on the surface of the cartridge are counted and tabulated for each target molecule.

Barcode	Counts	Identity
	3	XLSA
	2	FOXS
	1	INSULIN

# GOALS OF smRNA STUDY

- UNDERSTAND BIAS OF THE KITS
- SIZE RESTRICTION OF KITS
  - What comes down other than smRNA
- EASE OF USE / TIME
- REPRODUCIBILITY

# The Samples



## **MUR:** Miltenyi miRXplore Universal Reference ●

- 950 synthetic unmodified miRNAs
- Identical to human, mouse, rat
- 5'-phosphate moiety
- Equimolar ratios

## **MUR-D:** Miltenyi miRXplore Universal Reference + DICER (-) Cell Line ● ●

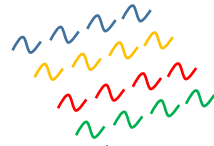
- No native miRNA
- Otherwise "normal" RNA background
- ATCC® CRL-3221™

## **HBR:** Human Brain Reference ●

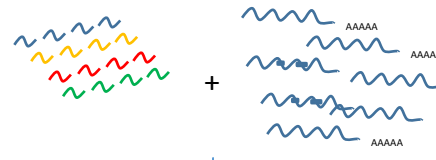
- Commonly used reference standard
- Unknown Catalog of miRNAs
- Unknown Molar ratio of miRNAs



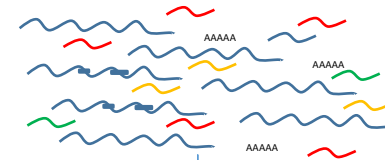
MUR



MUR-D



HBR

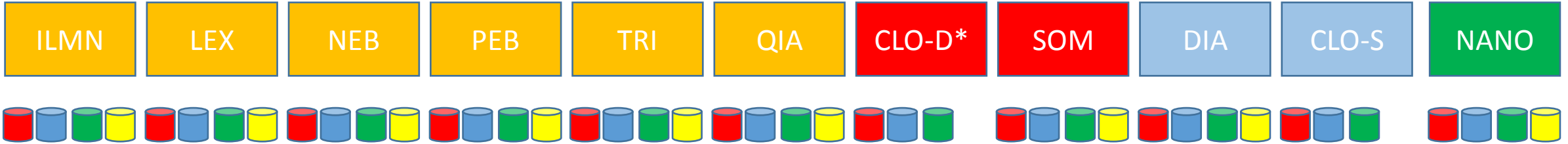


Sequential Ligation

Circularization

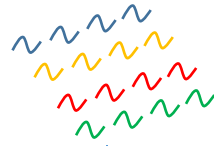
Template Switching

Ligation/  
Hybridization

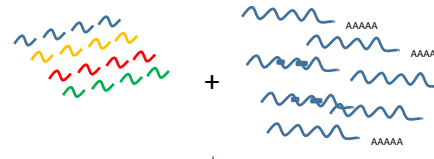


➤ EACH KIT TESTED AT 4 SITES – SAMPLES RUN IN TECHNICAL DUPLICATE ● ●  
 n=264 samples

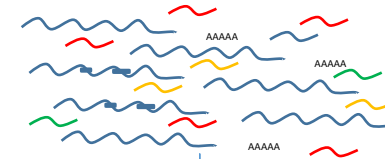
MUR



MUR-D



HBR



Sequential Ligation

Circularization

Template Switching

Ligation/  
Hybridization

ILMN

LEX

NEB

PEB

TRI

QIA

CLO-D\*

SOM

DIA

CLO-S

NANO

1ug

100ng

100ng

250ng

10ng

10ng

250ng

250ng

10ng

1ng

100ng

➤ EACH KIT TESTED AT 4 SITES – SAMPLES RUN IN TECHNICAL DUPLICATE

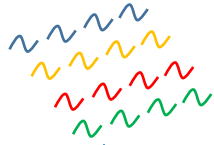


➤ VENDORS CONSULTED ABOUT RECOMMENDED INPUT AMOUNTS

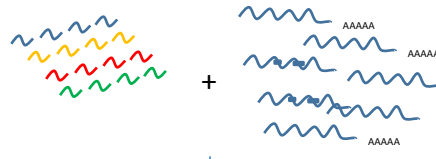


○ Call between vendor and sites before library preparation began

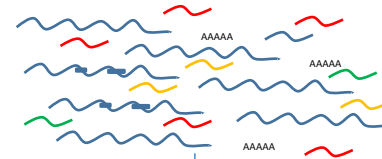
MUR



MUR-D



HBR



Sequential Ligation

Circularization

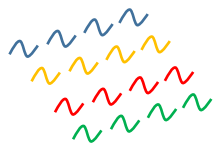
Template Switching

Ligation/  
Hybridization

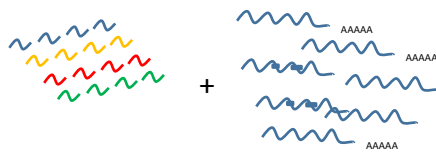
ILMN	LEX	NEB	PEB	TRI	QIA	CLO-D*	SOM	DIA	CLO-S	NANO
1ug	100ng	100ng	250ng	10ng	10ng	250ng	250ng	10ng	1ng	100ng



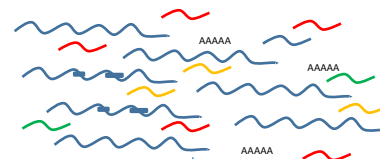
MUR



MUR-D



HBR



Sequential Ligation

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ILMN

LEX

NEB

PEB

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QIA

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SOM

DIA

CLO-S

NANO

1ug

100ng

100ng

250ng

10ng

10ng

250ng

250ng

10ng

1ng

100ng



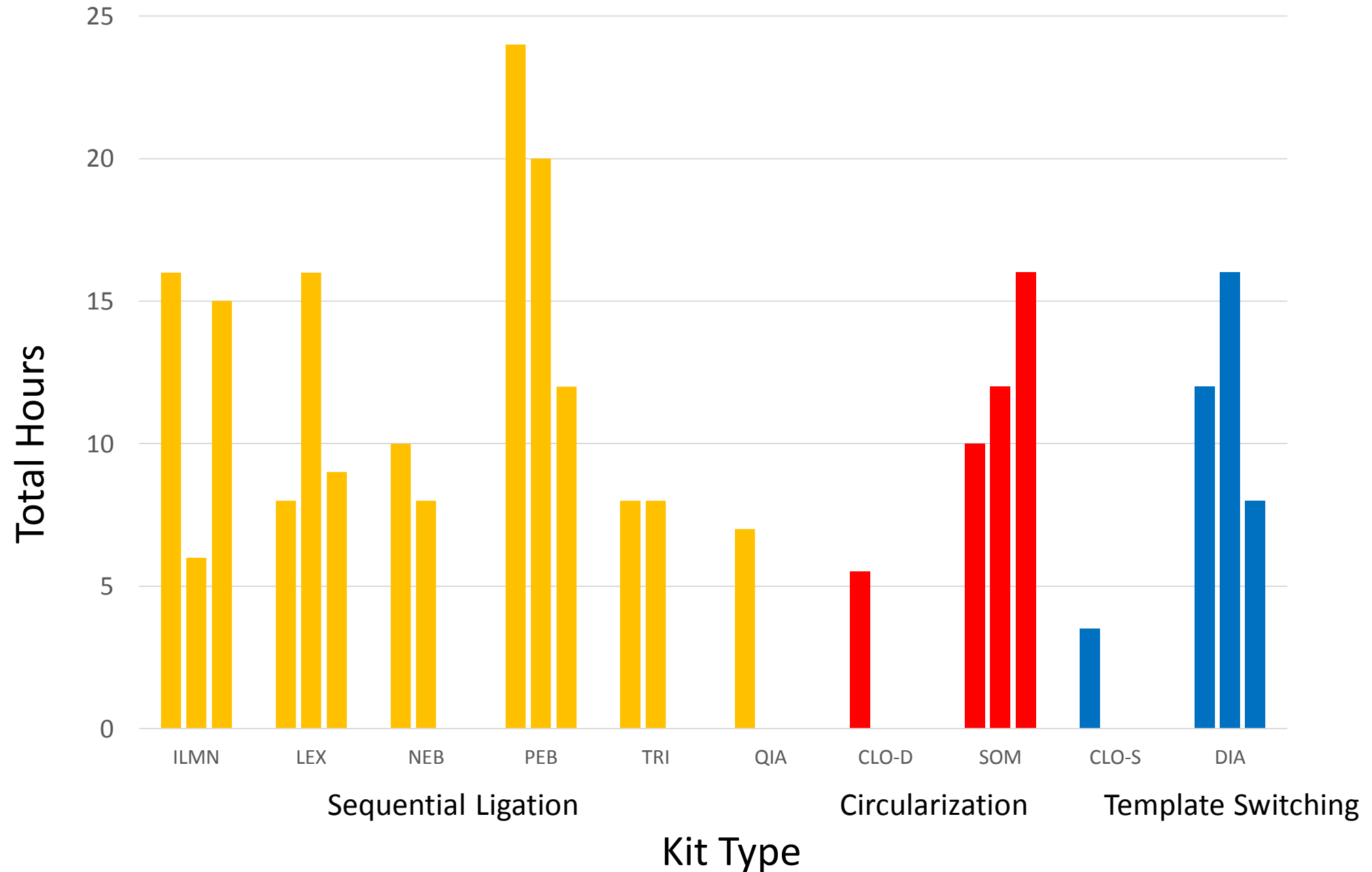
ILLUMINA NEXTSEQ

nCOUNTER

# GOALS OF smRNA STUDY

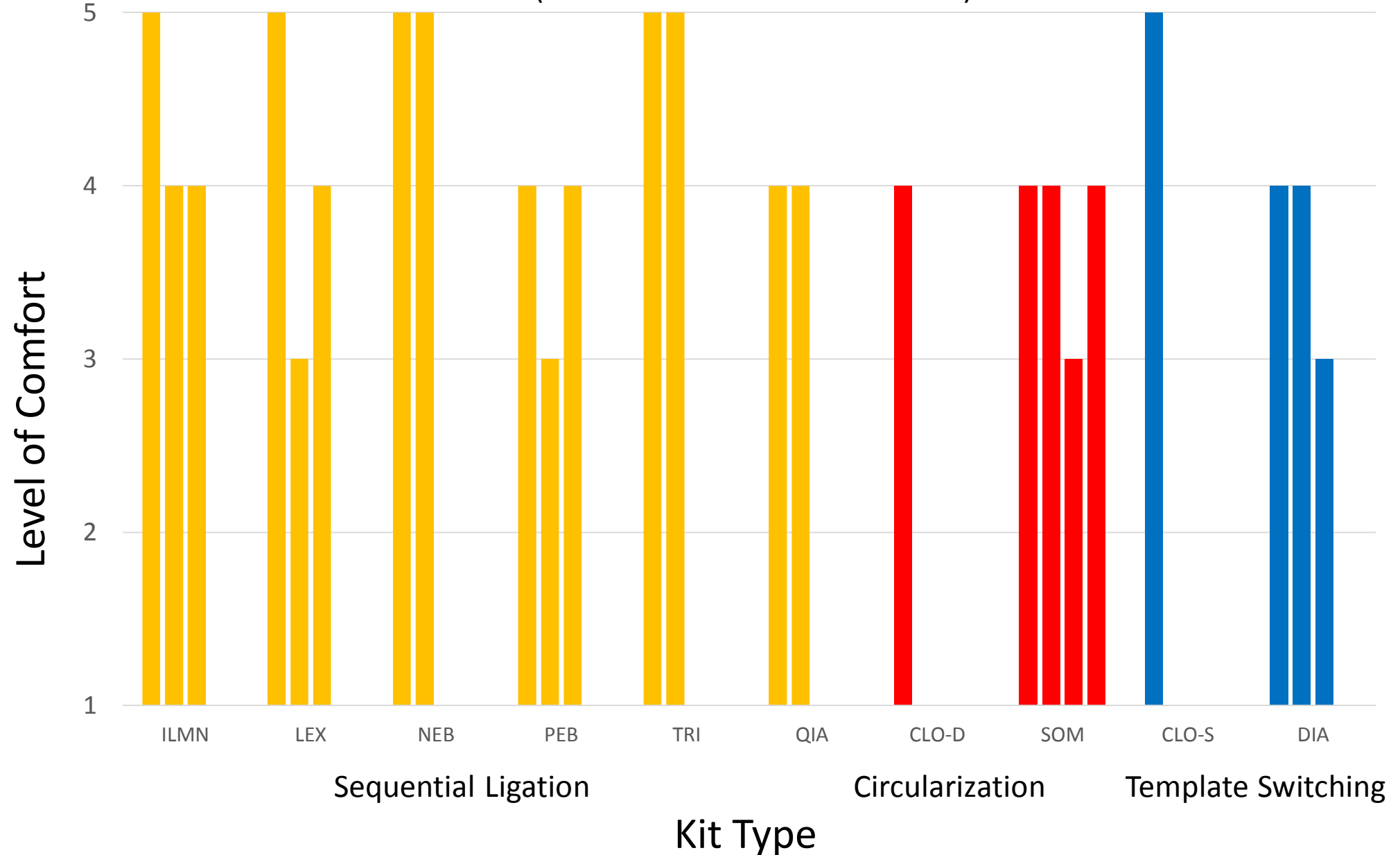
- UNDERSTAND BIAS OF THE KITS
- SIZE RESTRICTION OF KITS
  - What comes down other than smRNA
- EASE OF USE / TIME
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# Reported Time by Site to Complete a Library



# Reported Level of Comfort using the the kit by Site

(1 least and 5 most comfortable)

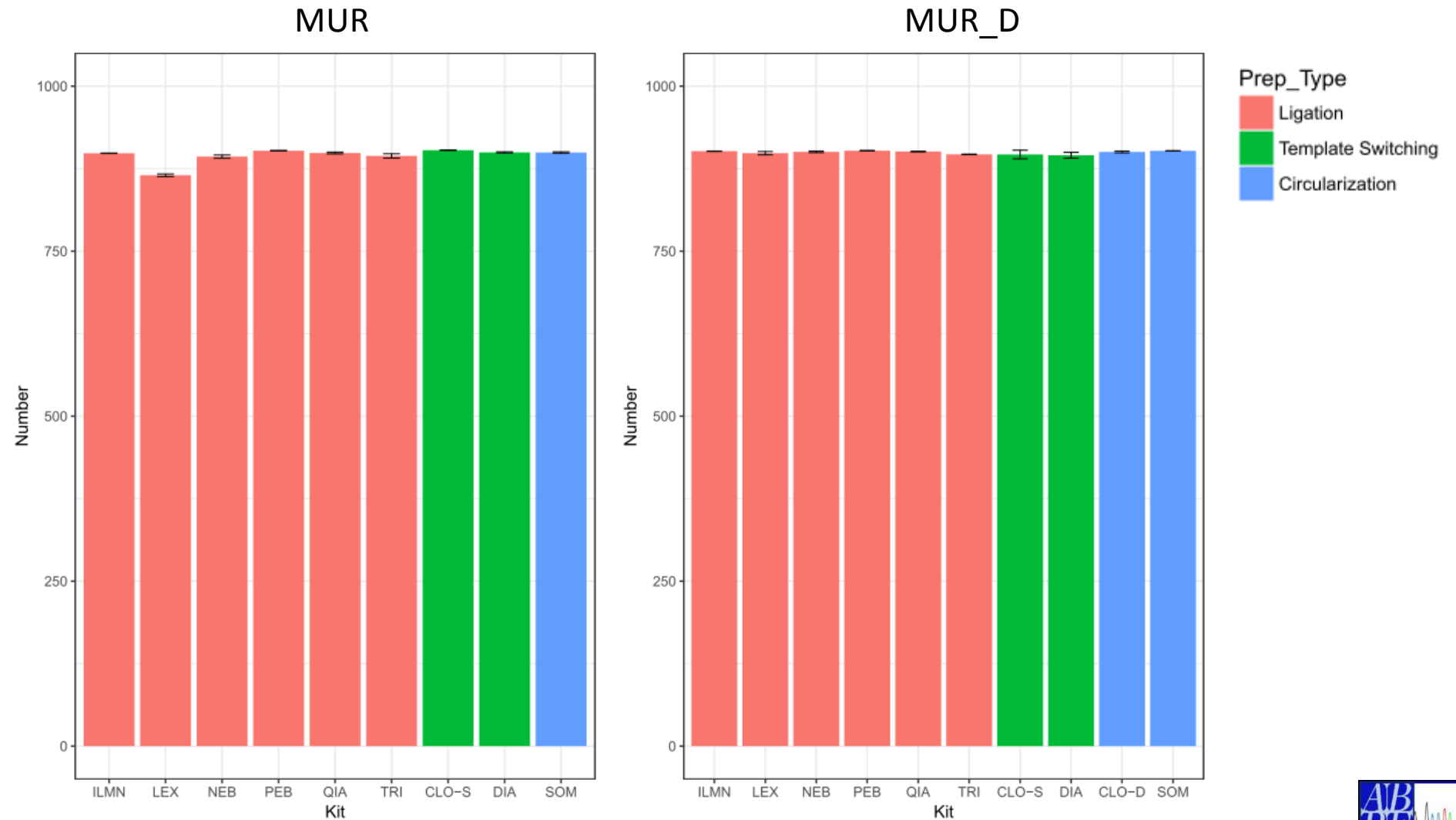


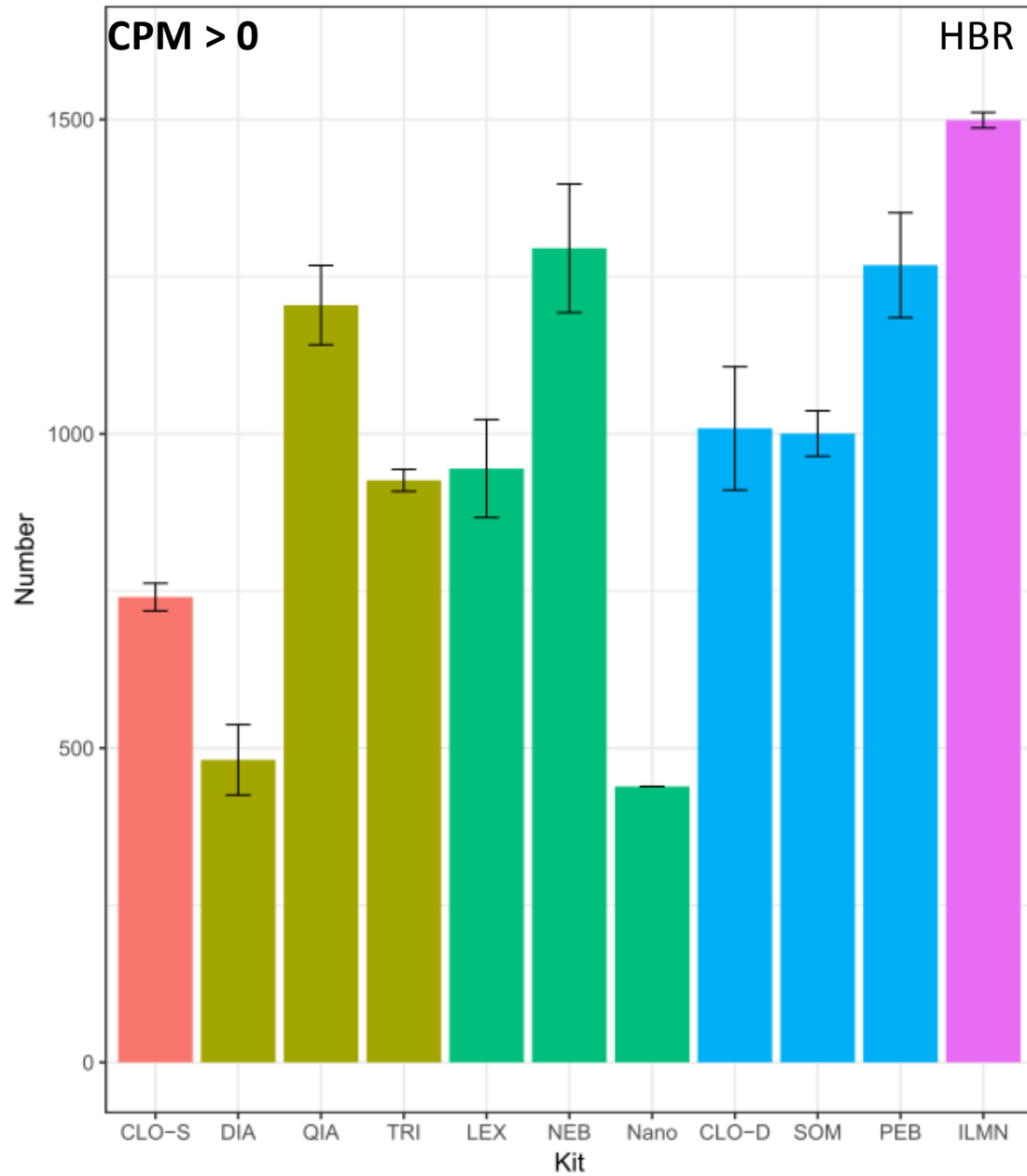
# FAILED LIBRARIES

CHEMISTRY	Number of Libraries	Dropped from Analysis	-- on us --
Illumina	24	2 of 12	
Lexogen	24	3 of 12	
NEB	24		
BIOO	24		
TriLink	18		1 site returned late
Qiagen	24		
Rubicon (CLO-D)	18	4 of 9 (all MUR samples)	1 site returned late
Diagenode	24	3 of 12	
Clontech (CLO-S)	18	4 of 9	1 site returned late, 1 set MUR reps not returned due to failure
Somagenics	24		
Nanostring	24		



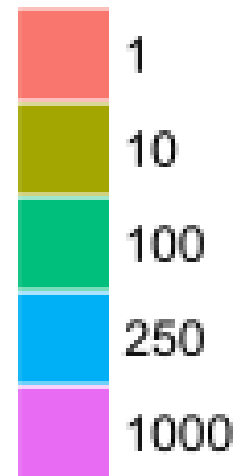
# miRNA DETECTION



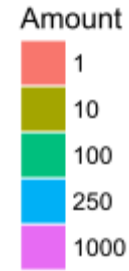
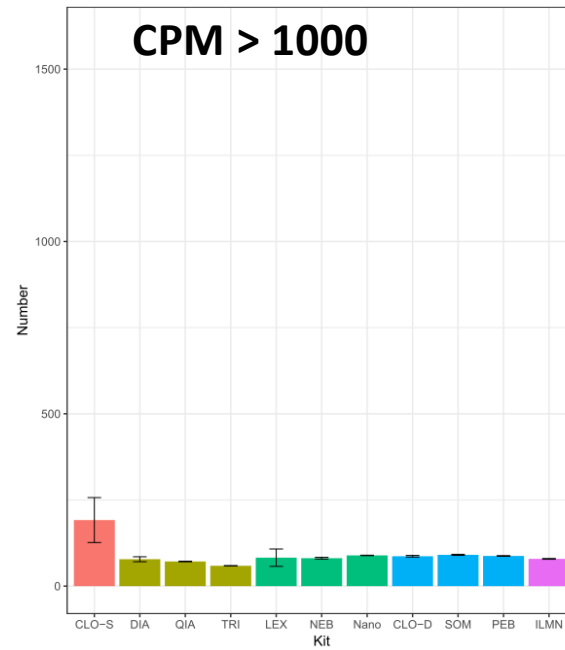
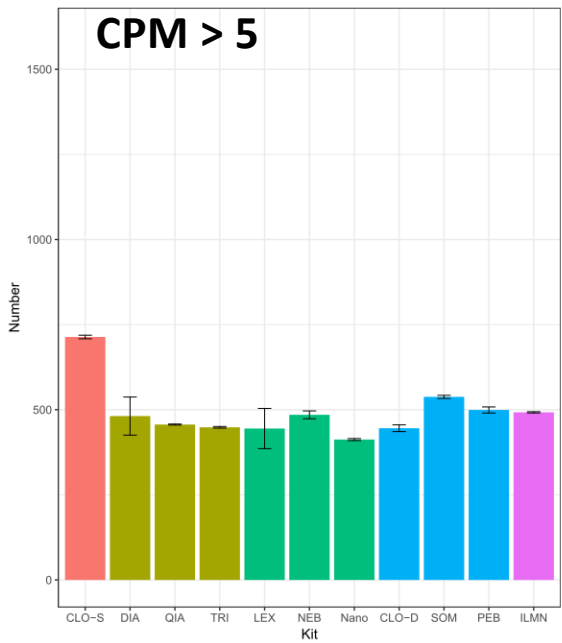
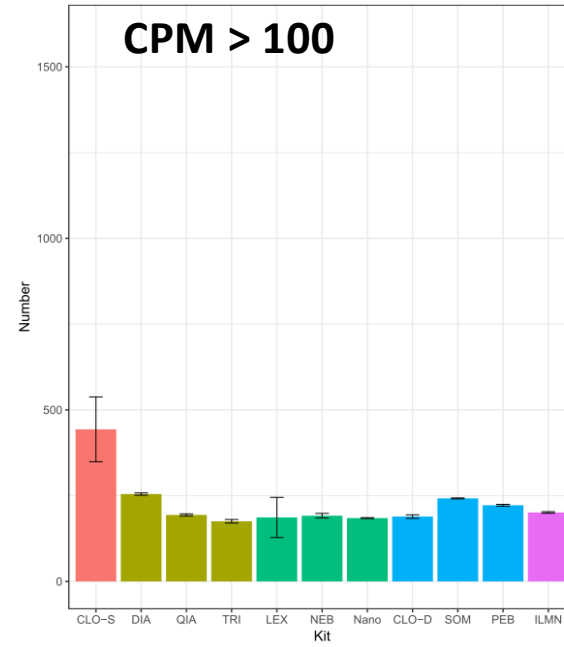
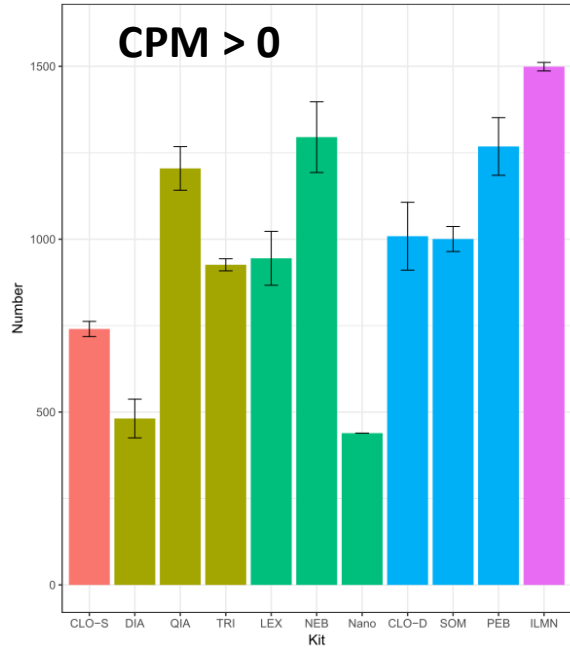


## Number of miRNA detected in Brain Reference

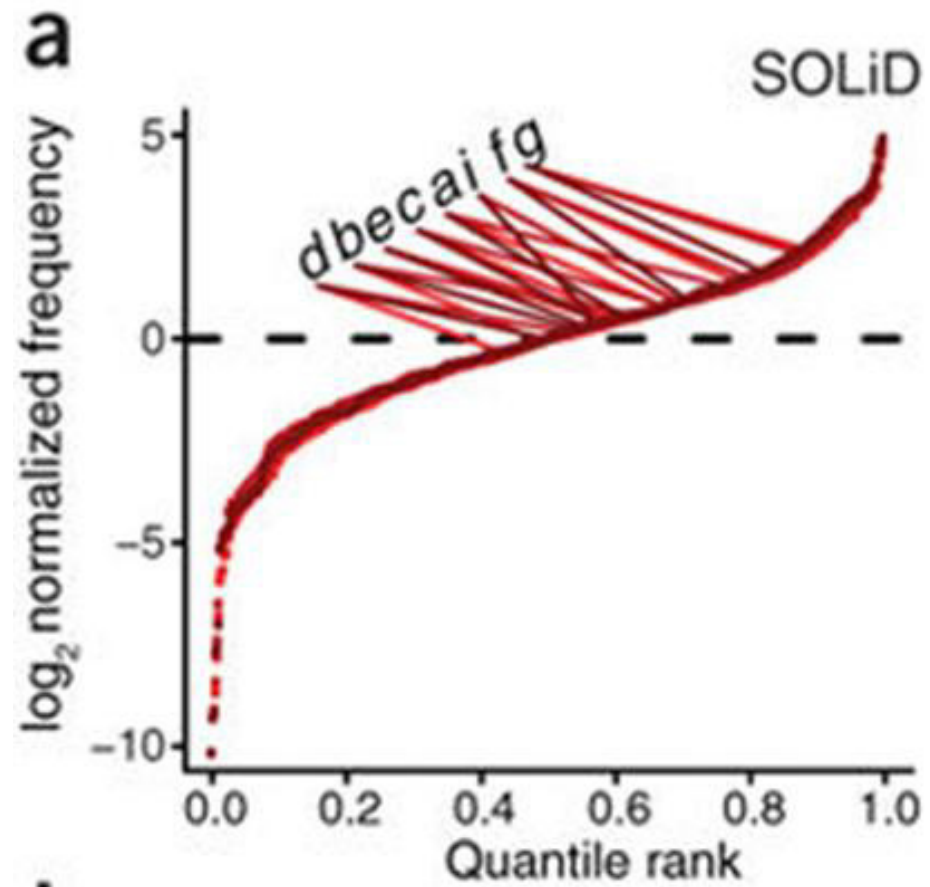
Input Amount



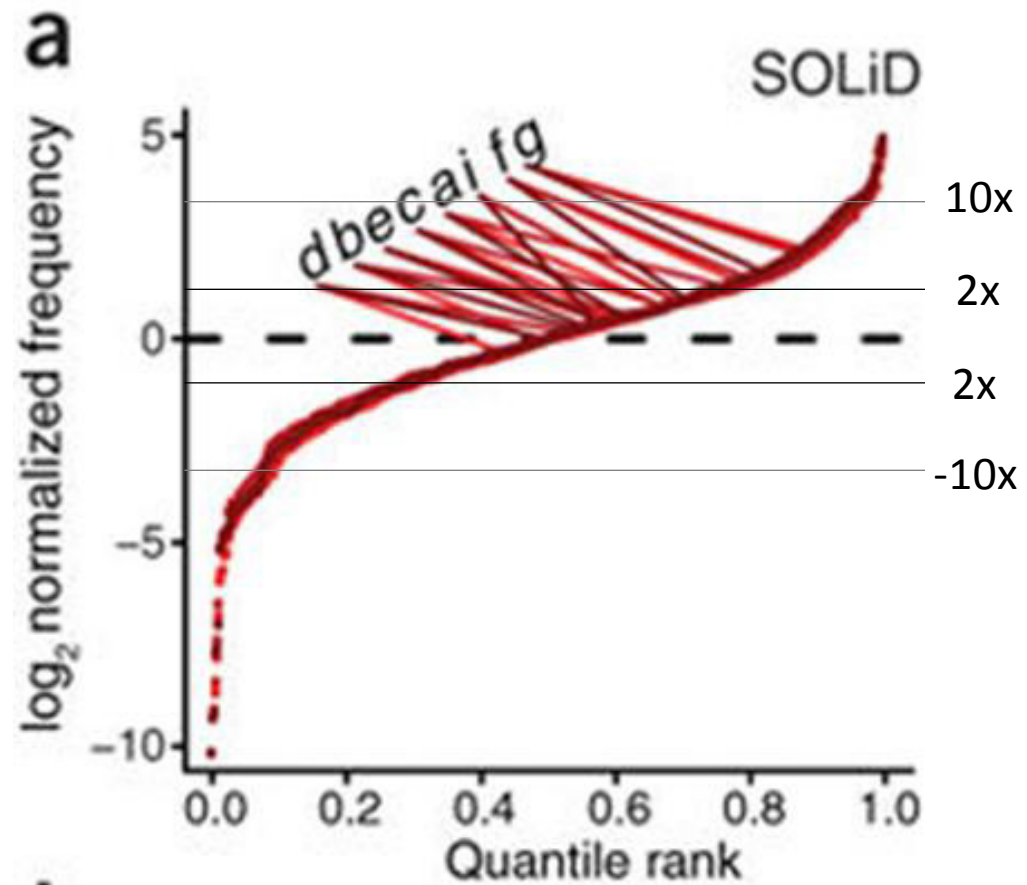
# Number of miRNA detected in Brain Reference



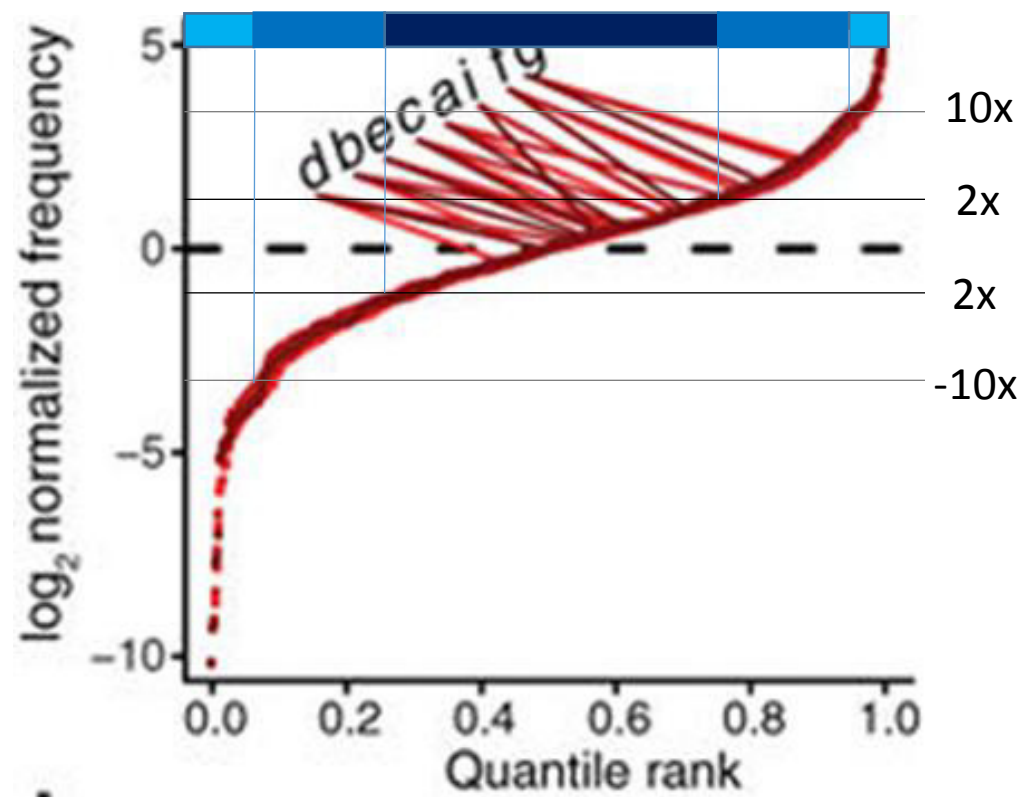
# miRNA BIAS



# miRNA BIAS



# miRNA BIAS

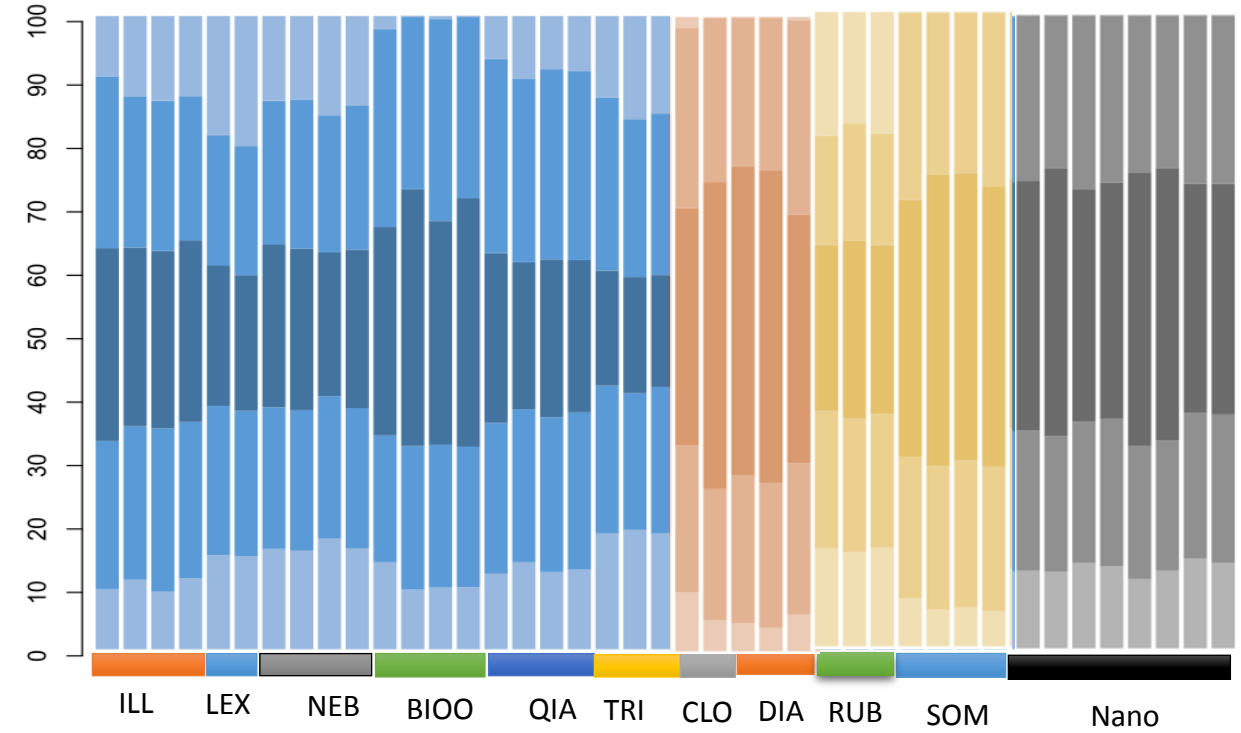
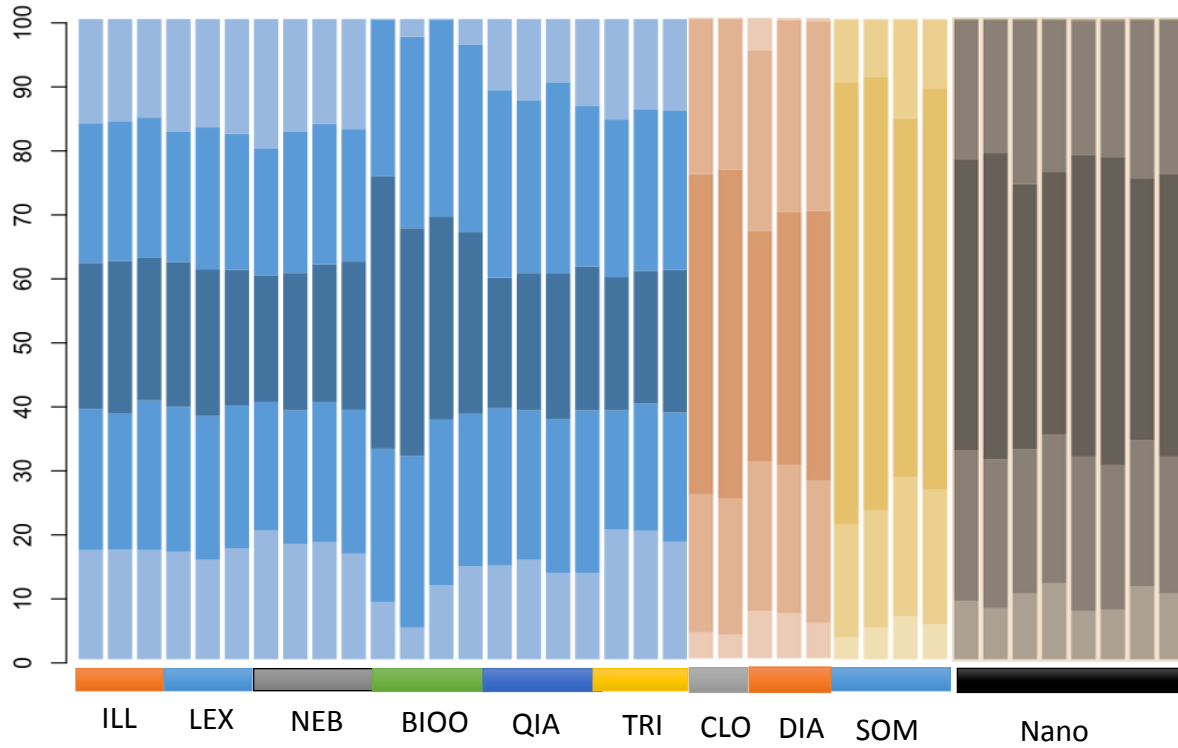




Percentage of miRNA for 5 categories (<10x down, 10-2x down, -2x - +2x, 2-10x up, >10x up)

MUR

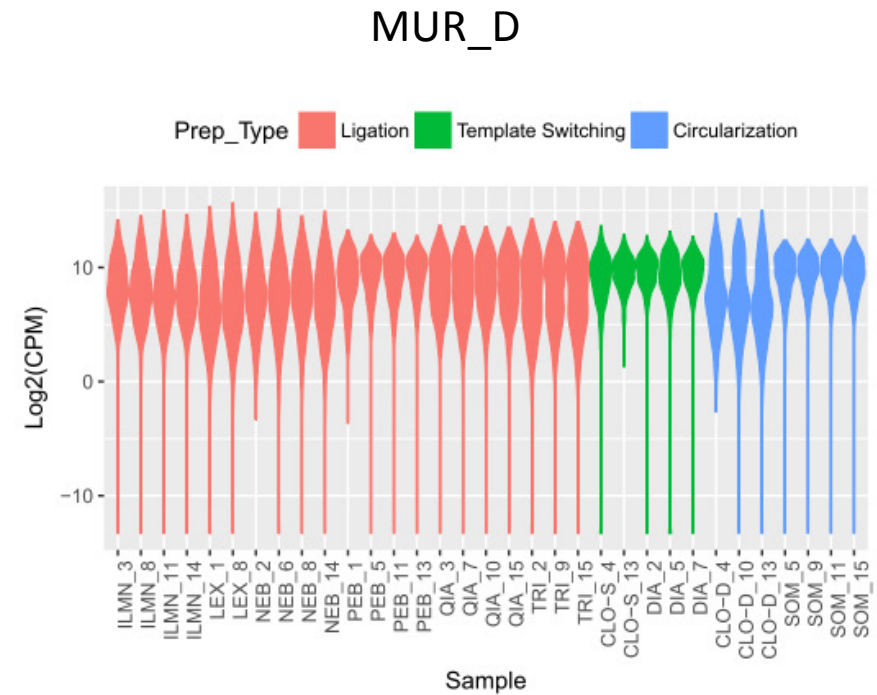
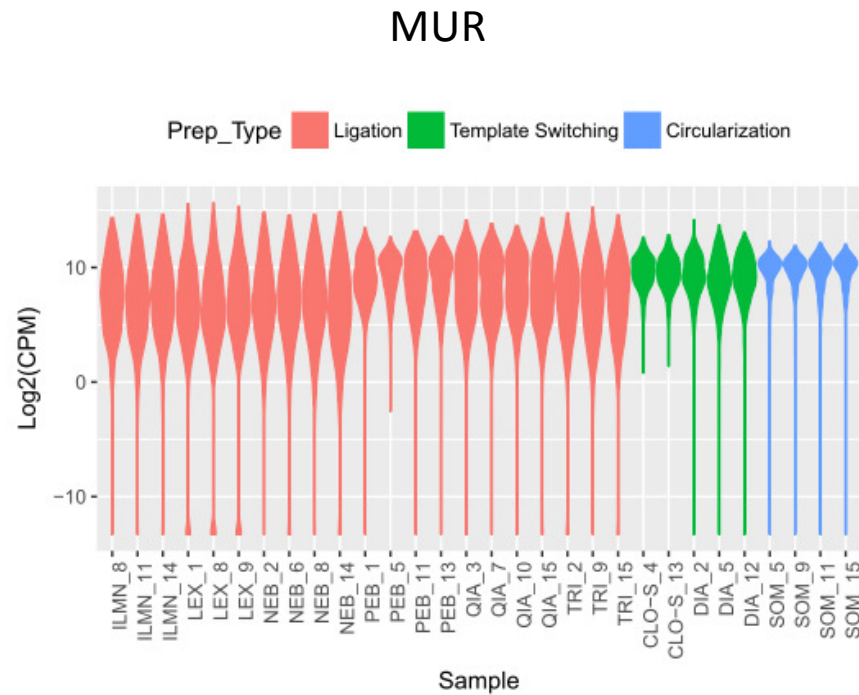
MURD



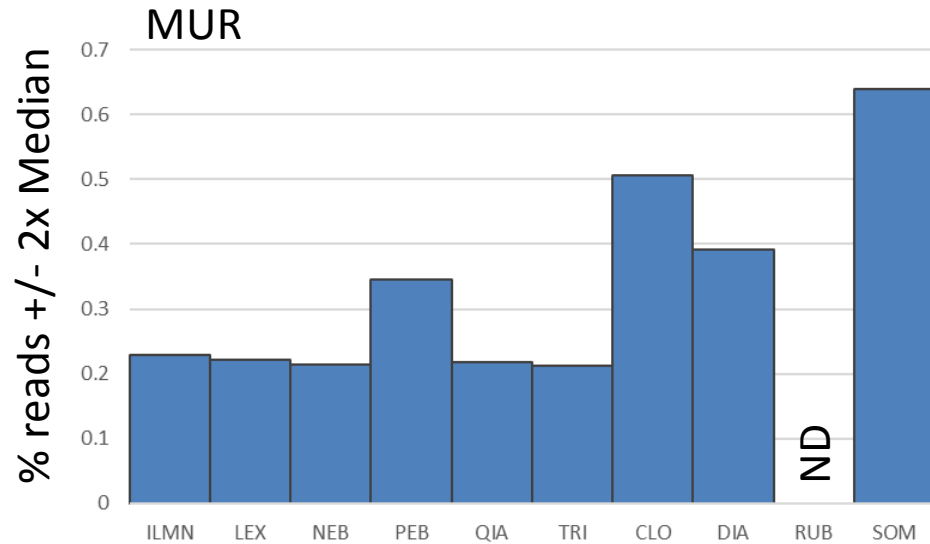
- Percentage of < 10x down
- Percentage of 10-2x down
- Percentage of within -2x and +2x
- Percentage of 2-10x up
- Percentage of >10x up



# Violin plots for the miRNAs with log2 transformed CPM



# miRNA BIAS CONCLUSIONS

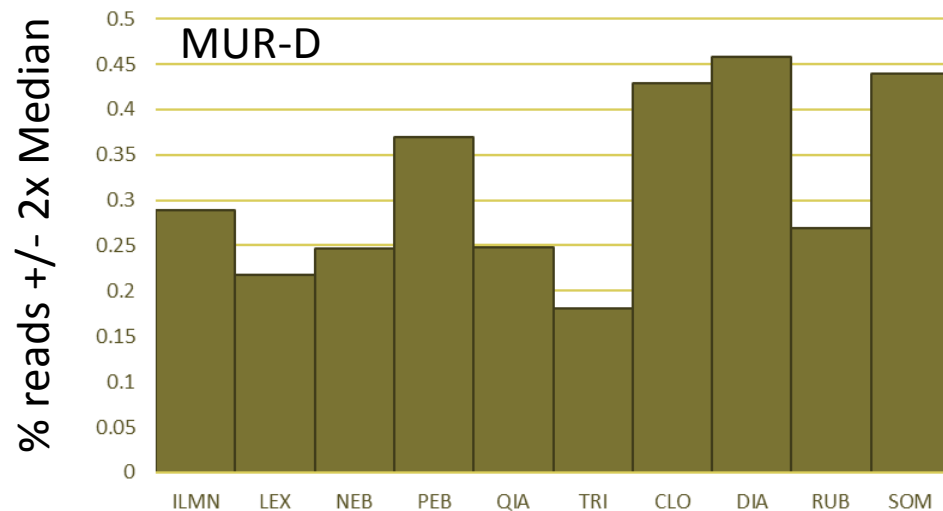


➤ ALL kits show bias. NO kit showed even 50% of miRNAs within +/-2x in a native context.

➤ Circularization + polyA do reduce the bias more alone than in context of totalRNA

➤ B100 shows slightly less bias than other ligation based methods

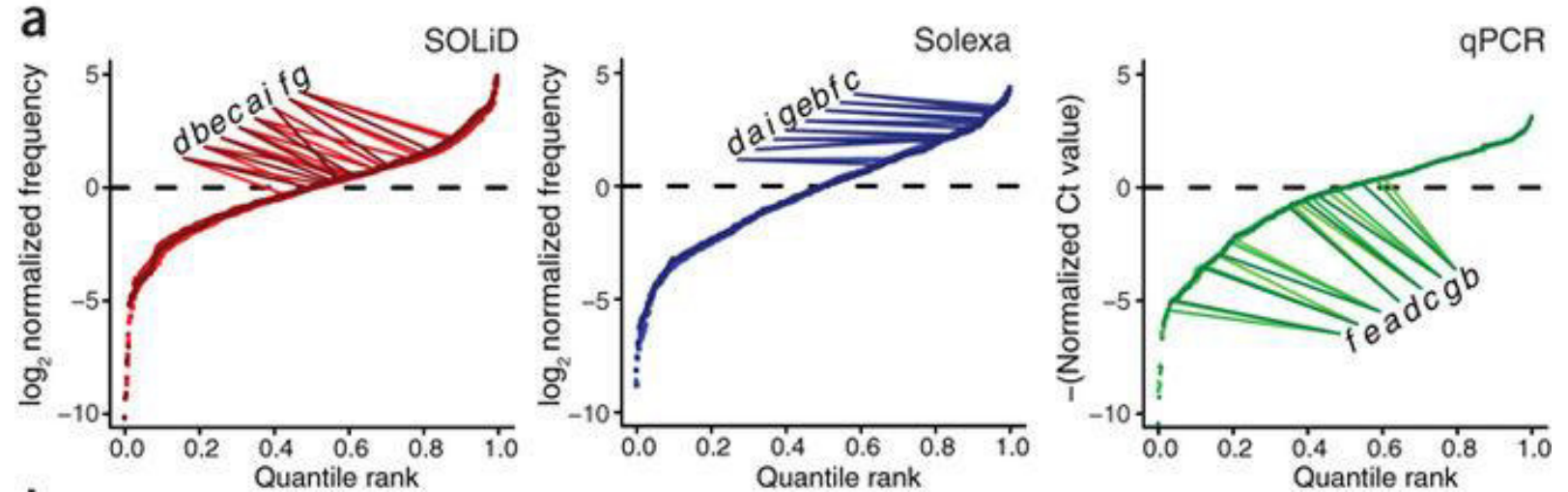
➤ Newer methods and B100 chemistry show almost no jackpotting



# RELATIVE DETECTION OF SMALL RNAs

Bias is reproducible – running the same kit twice gives the same result

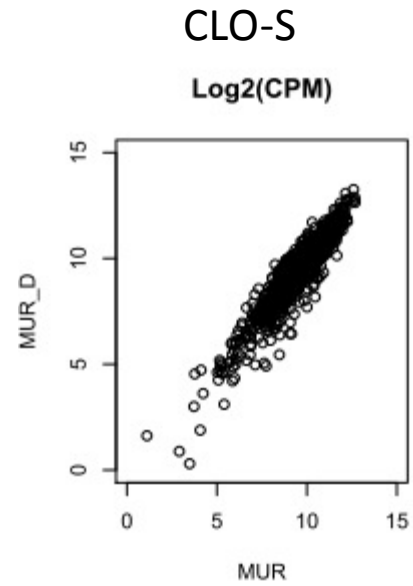
\* RELATIVE values are robust, ABSOLUTE values are not



Linsen et al., Nature Methods 2009

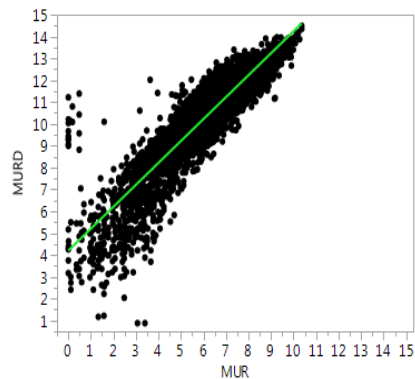
# REPRODUCIBILITY OF BIAS

Log2 transformed CPM values were used.



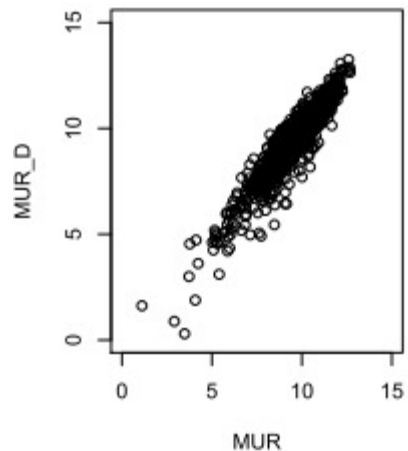
# REPRODUCIBILITY OF BIAS

NANO



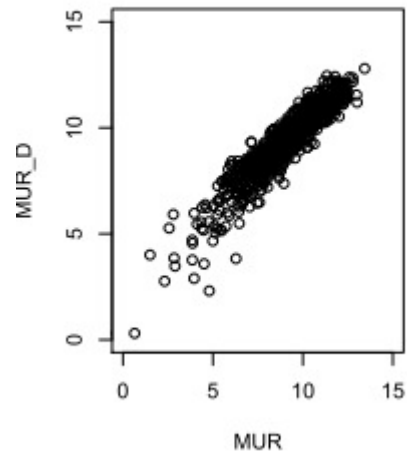
CLO-S

Log2(CPM)



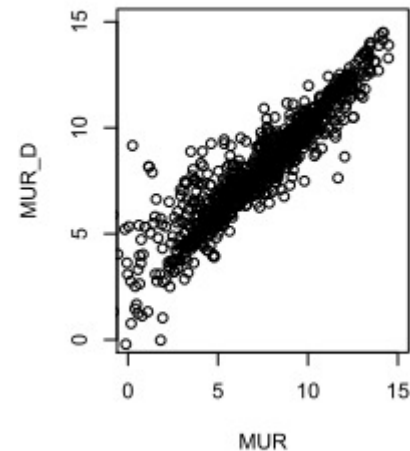
DIA

Log2(CPM)



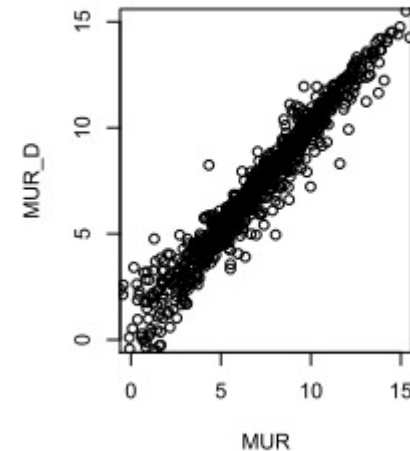
ILMN

Log2(CPM)



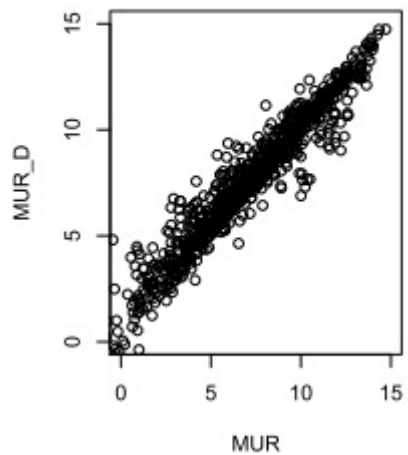
LEX

Log2(CPM)



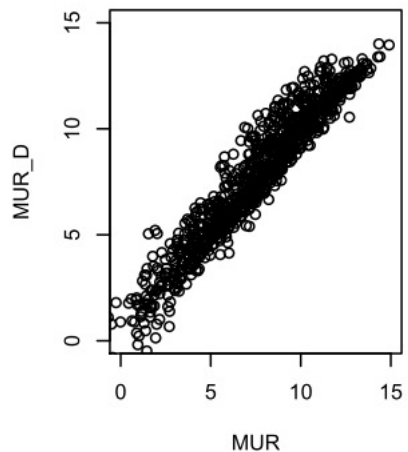
NEB

Log2(CPM)



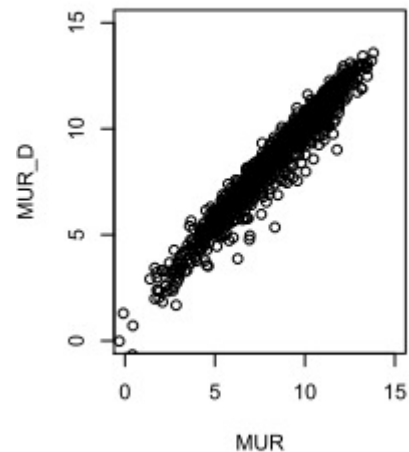
TRI

Log2(CPM)



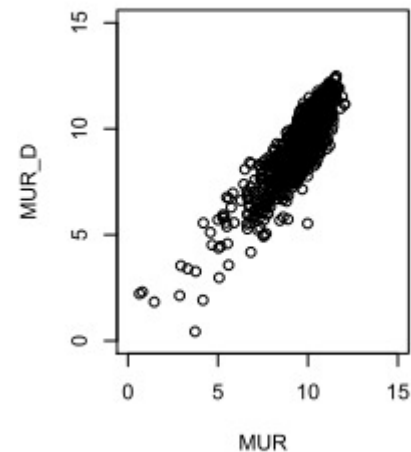
QIA

Log2(CPM)



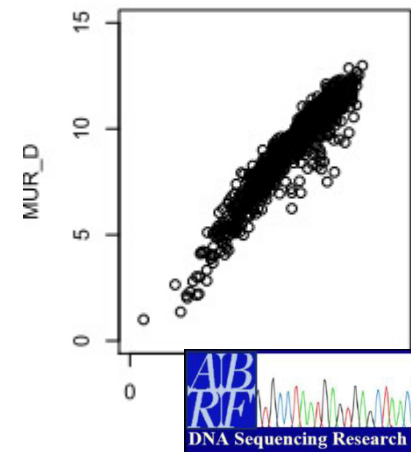
SOM

Log2(CPM)

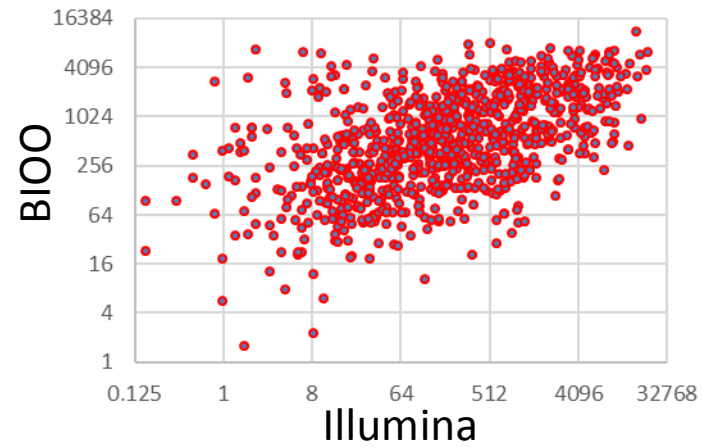
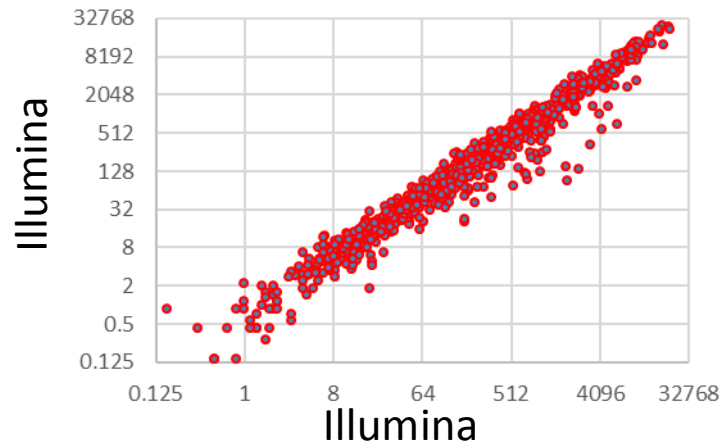


PEB

Log2(CPM)



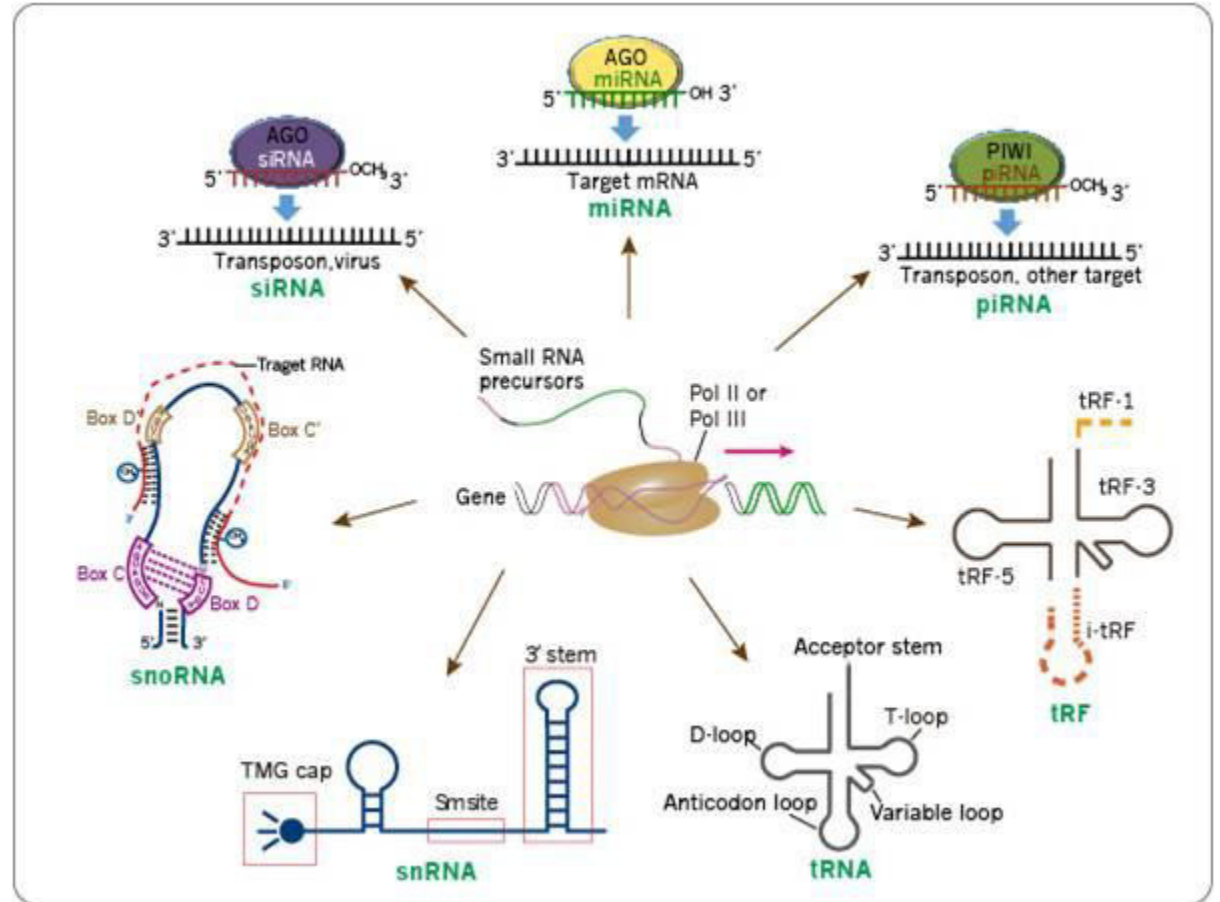
# Kit-to-kit show very different results:



# GOALS OF smRNA STUDY

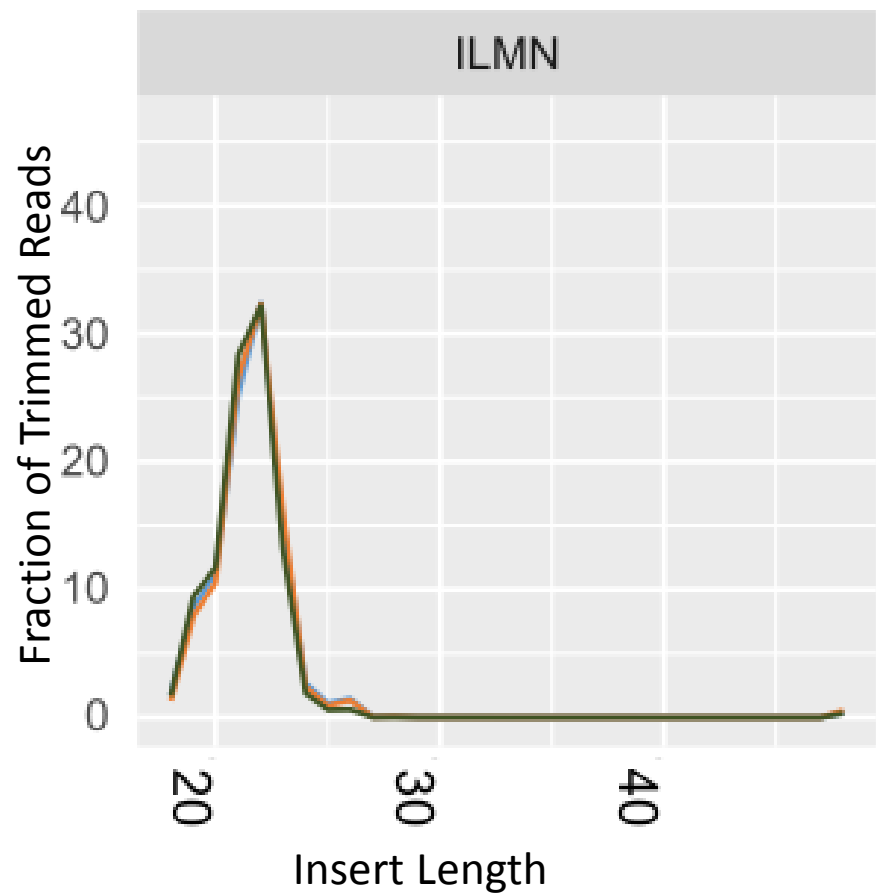
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# Small RNA

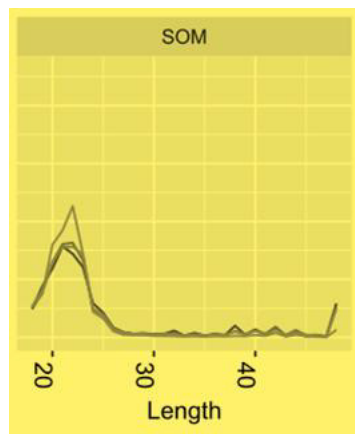
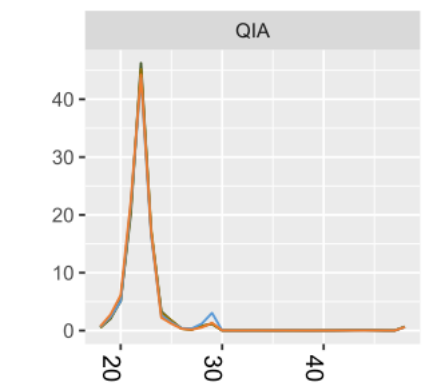
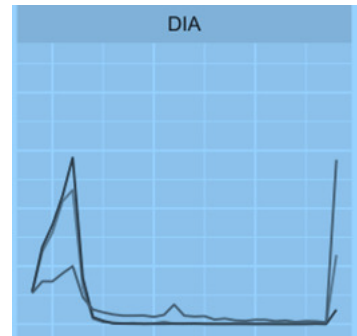
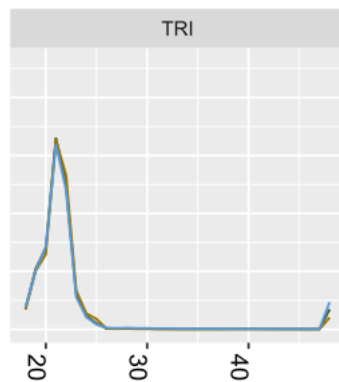
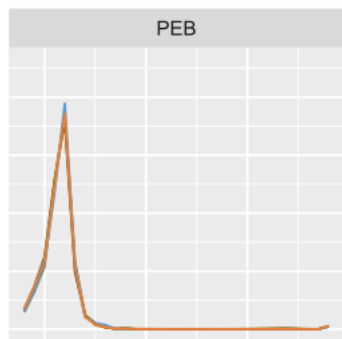
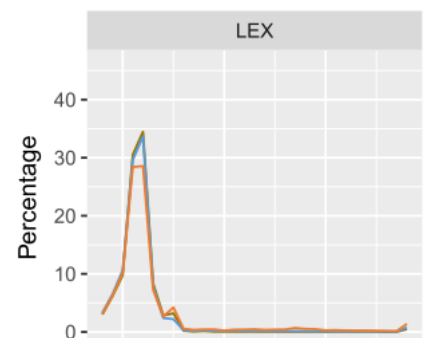
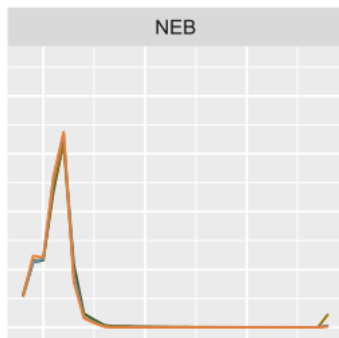
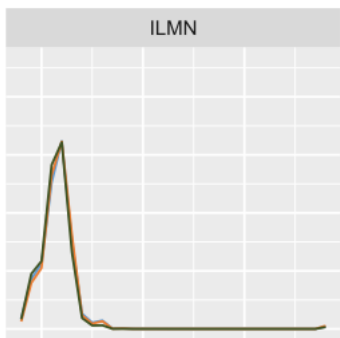
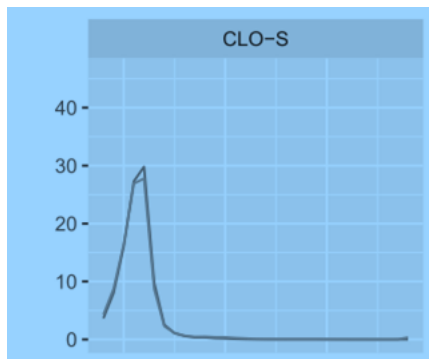




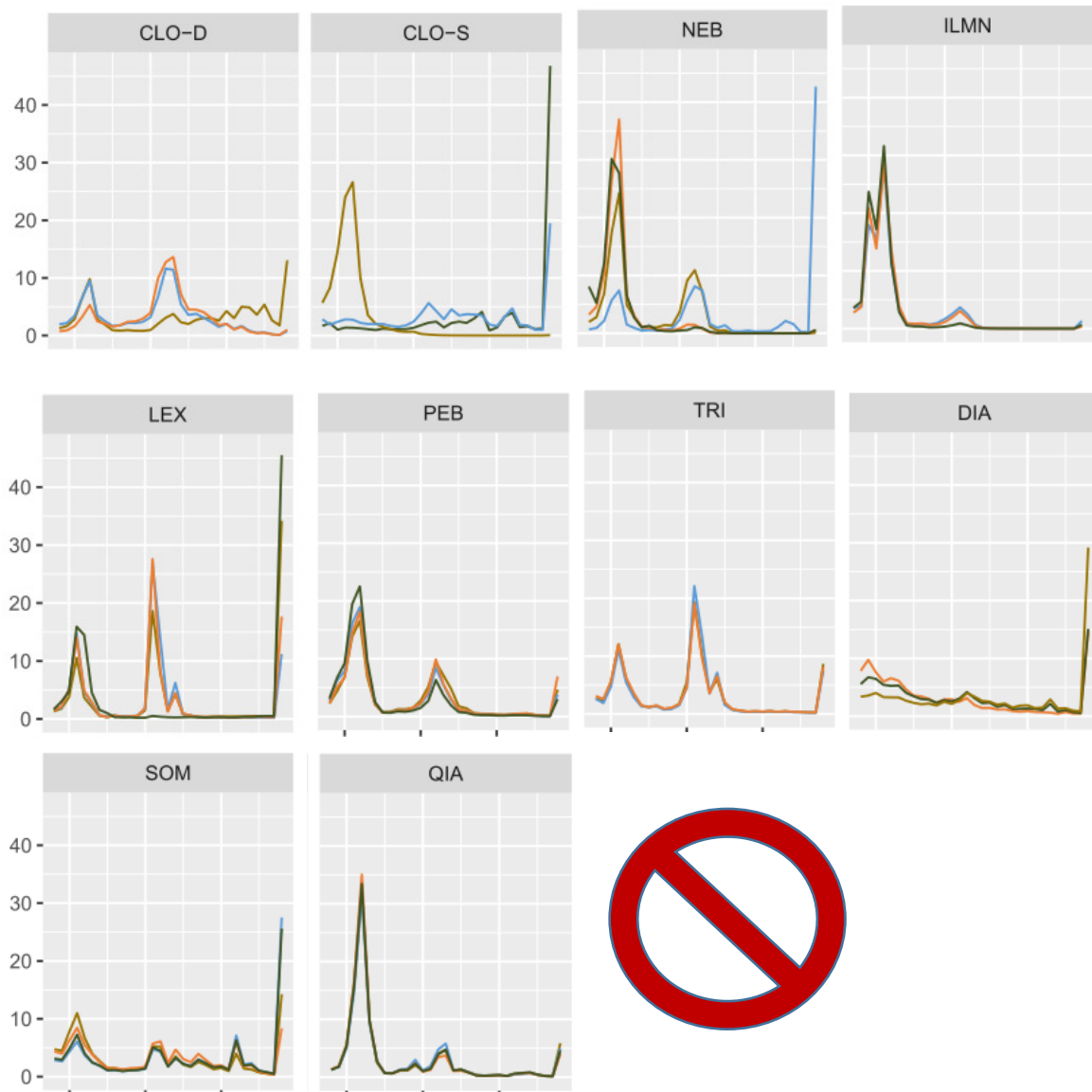
# Read length distribution for MUR samples



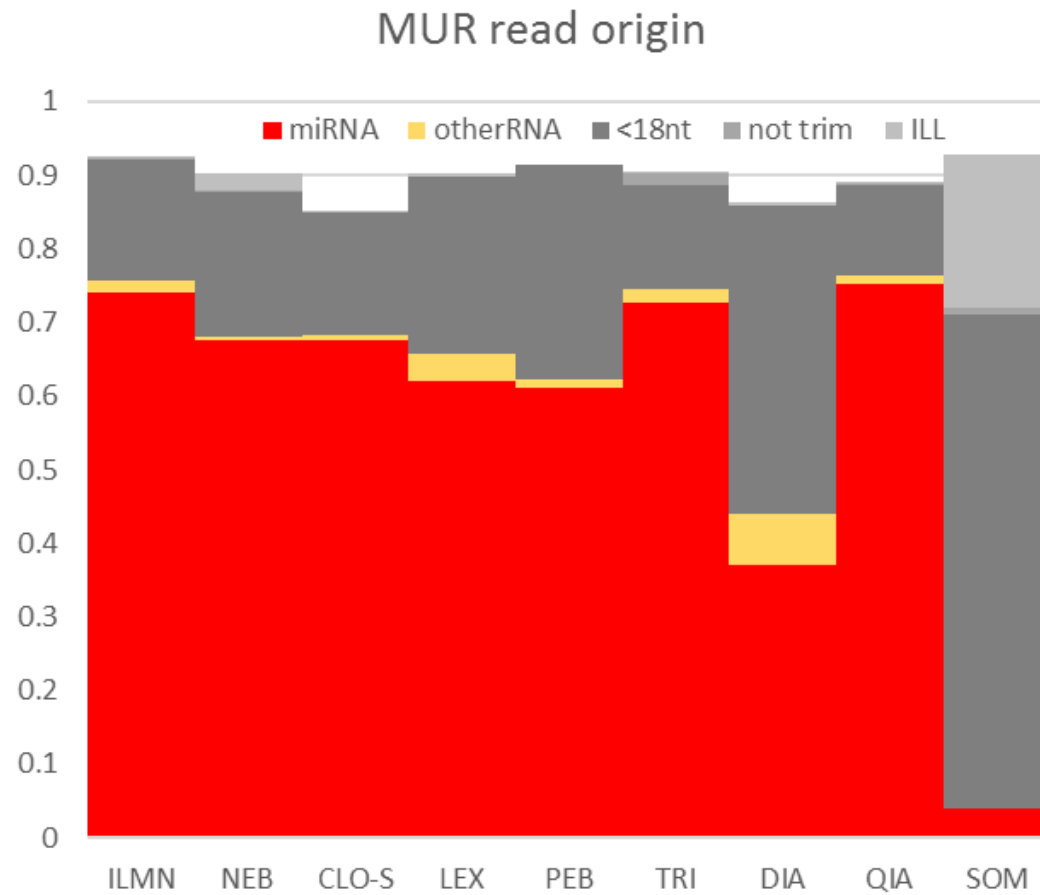
# Read length distribution for MUR samples



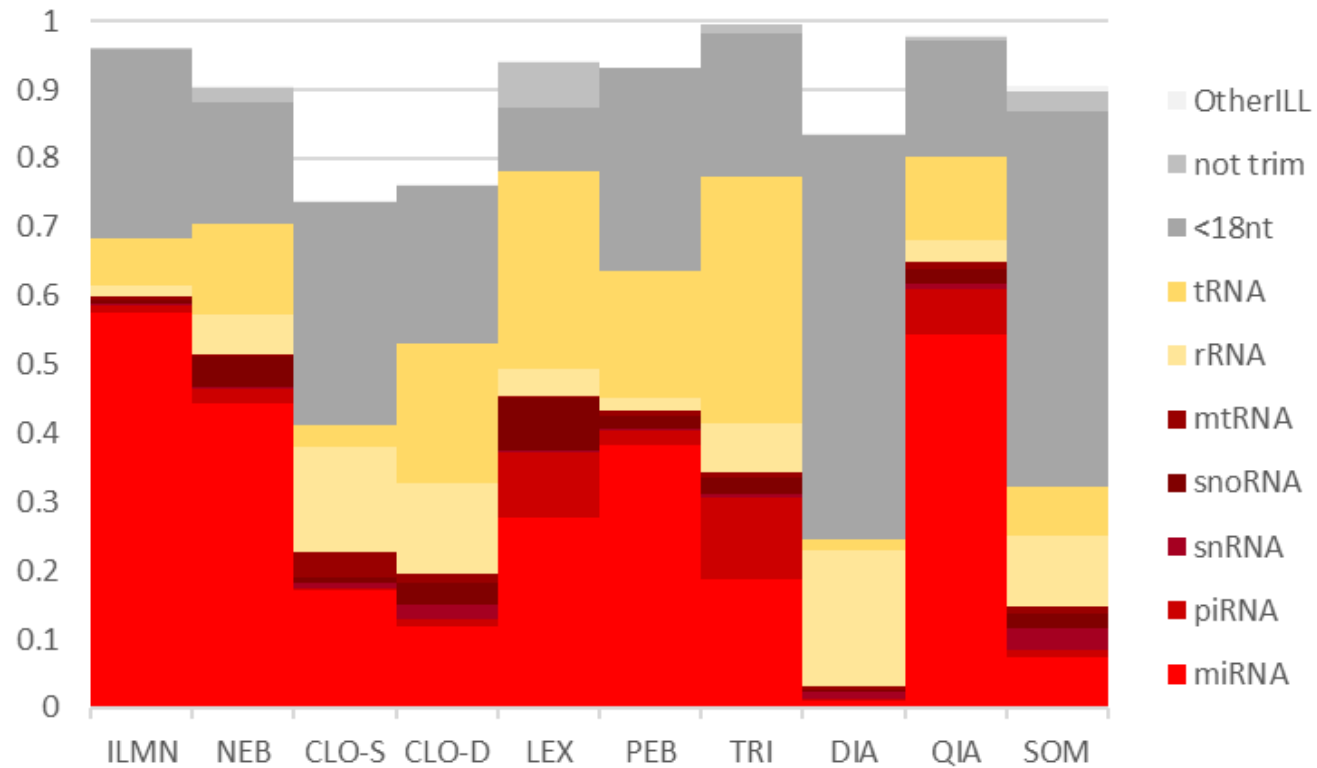
# Read length distribution for HBR samples



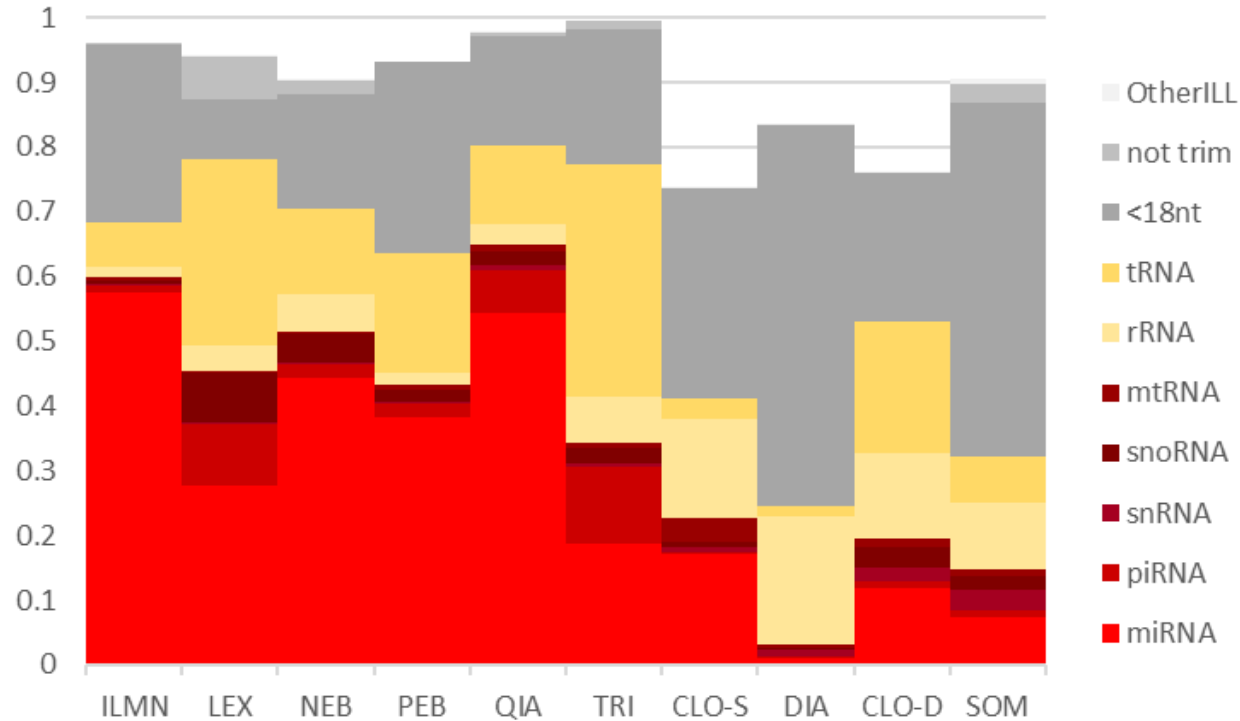
# MUR Read mapping



# HBR Read mapping



# HBR Read mapping



- Observe higher number of primer artifacts with newer chemistries
- Primer artifacts are observed in all chemistries
- Some chemistries may be unsuitable for longer small RNAs (eg Qiagen)

# GOALS OF smRNA STUDY

- UNDERSTAND BIAS OF THE KITS
- SIZE RESTRICTION OF KITS
  - What comes down other than smRNA
- EASE OF USE / TIME
- REPRODUCIBILITY

# CONCERNS / CAVEATS / NEXT DIRECTIONS

Depends heavily on the Miltenyi controls

Understand the biases observed

Unmapped reads

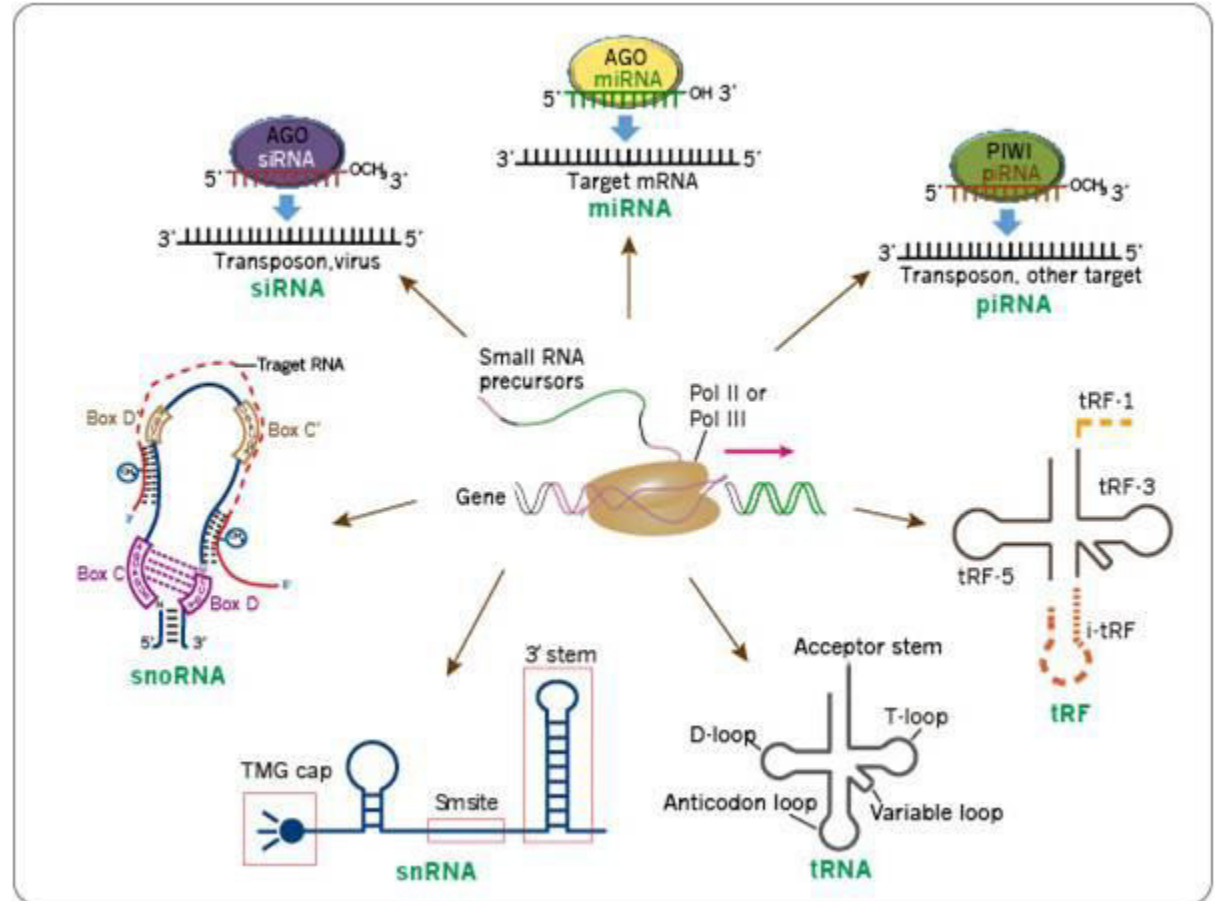
Finish user information gathering



# SUMMARY

The kits have very different strengths

- Discovery
- Evenness / lack of bias
- Low input
- Automation friendliness
- miRNA focus vs broad focus



# SUMMARY II

- All chemistries do work to produce smRNA libraries
- Significant bias is present in all kits
  - Circularization, polyA and B100 significantly reduce jackpotting.
- Correlation between input and de novo detection
- Circularization and PolyA preparations currently have lower fraction of small RNA reads.
  - Issues are largely technical and likely resolvable with additional protocol optimization

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## Experiments

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