Working with RNA in the Lab

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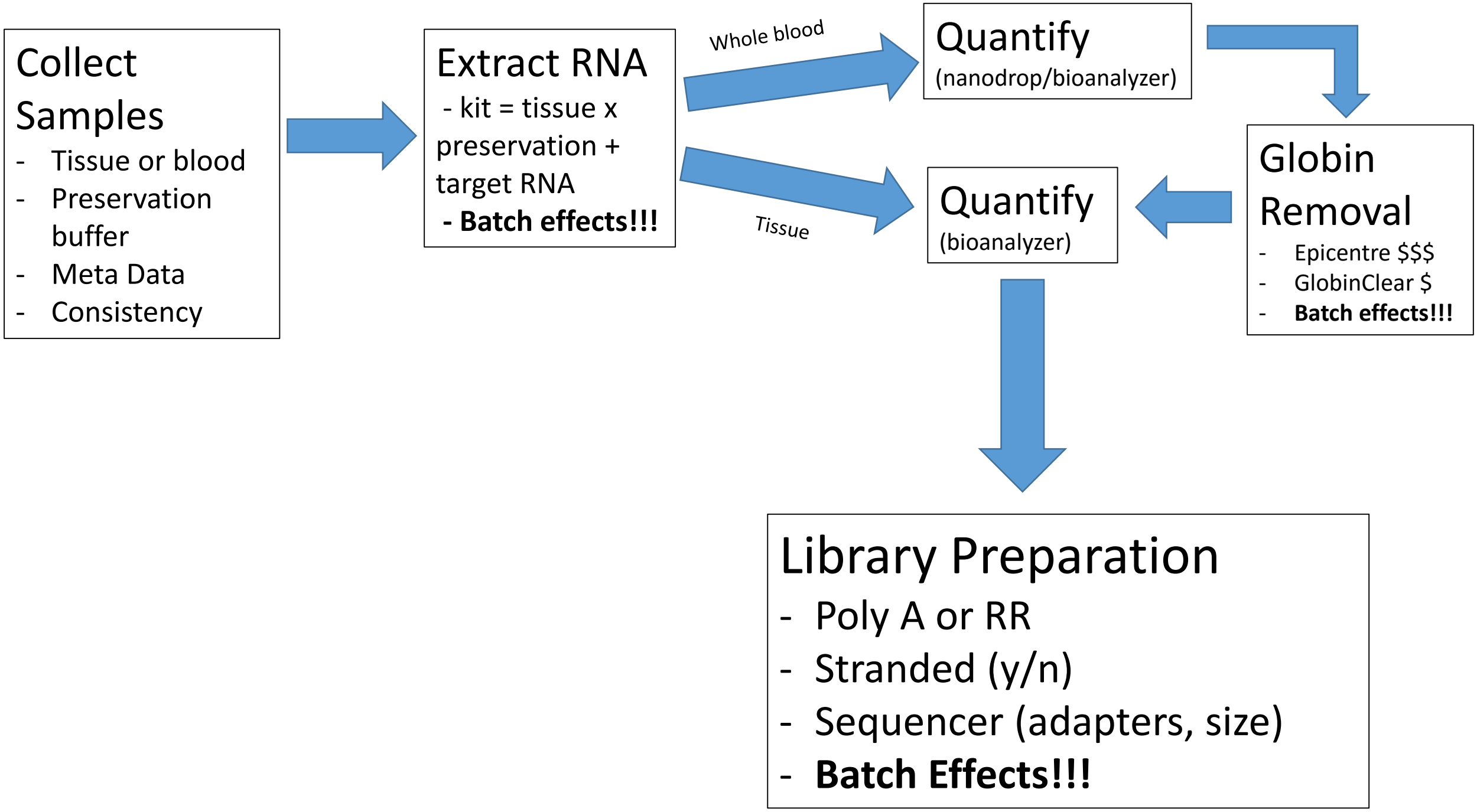
OUTLINE

- Sample size, study design and target RNA
- Batch effects (review)
- Collecting and Processing Samples:
  - Sampling collection and preservation
    - Collection should be as consistent as possible across samples and all meta data collected
    - Preservation: time sensitive and tissue specific
  - Extraction
    - Kits for various tissues
    - Additional steps (globin removal)
  - Quantification (bioanalyze at each step)
  - RT-qPCR
  - Library Prep (stranded vs. unstranded, sequencing platform, poly-A or ribosomal depletion)
  - Sequencing (paired vs single end, length of reads, coverage, pooling, lane effects)
  - Single Cell cDNA Libraries


Collect Samples
- Tissue or blood
- Preservation buffer
- Meta Data
- Consistency
Extract RNA
- kit = tissue x preservation + target RNA
- Batch effects!!!
Whole blood
Tissue
Quantify
(nanodrop/bioanalyzer)
Quantify
(bioanalyzer)
Globin Removal
- Epicentre $$$
- GlobinClear $
- Batch effects!!!
Library Preparation
- Poly A or RR
- Stranded (y/n)
- Sequencer (adapters, size)
- Batch Effects!!!

Study Design: Statistical Power

Statistical power is the ability to observe a significant effect on gene expression (given that the effect is biologically real)

- Depends on your sample size and the magnitude of the effect
  - BIG effect, SMALLER sample size is okay
  - **SMALL effect, need LARGE sample size**

→ Noisy data can limit the ability to detect an effect

→ Sampling natural populations introduces a lot of noise!

Levels of Control in Experimental Design

Extremely controlled: studies can be carried out on cultured cells from your species

Well-controlled: common-garden type setting (e.g. expose coral larvae to different pH water in the lab)

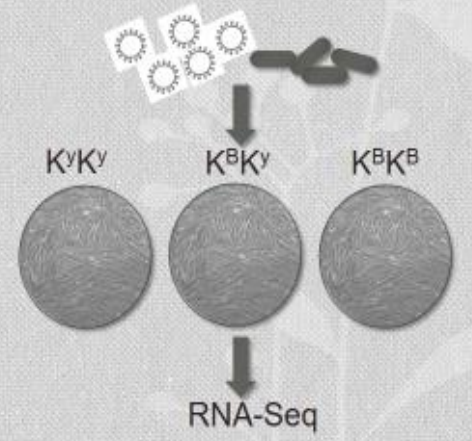
Wild populations: much harder to control factors, need a large sample size and as much metadata as possible

Cell culture allows experimentation

N = 6

Question: Wolves have different genotypes at the K locus. Does this impact their immune system?

Approach: Challenge wolf cells of different genotypes and compare immune responses with RNA-Seq.



RNA-Seq to unravel the genetics of bird migration in Swainson's thrushes

N = 20

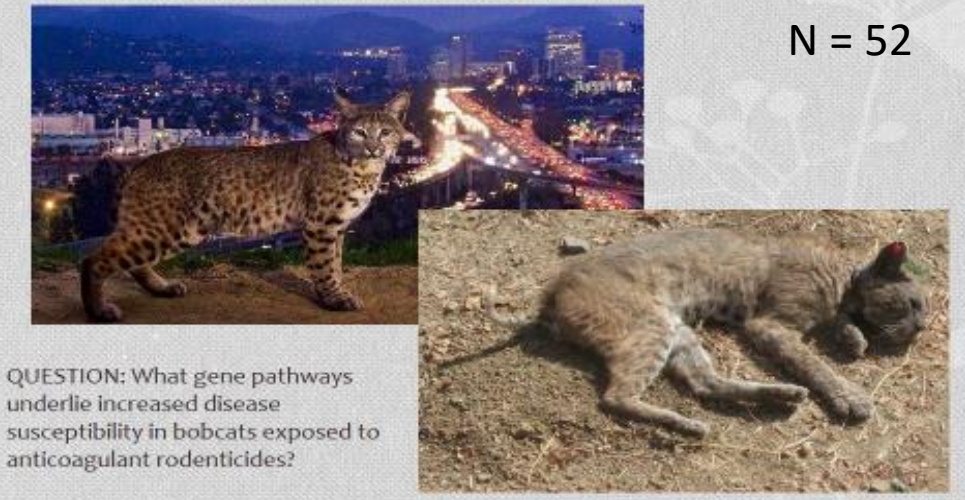
	Summer 2010	Fall	Winter	Spring	Summer	Fall
Coastal	Birds maintained in captivity				(N=5)	(N=5)
Continental	Birds maintained in captivity				(N=5)	(N=5)

The importance of age in structuring gene expression profiles in Yellowstone Wolves

N = 28

Mange/Rodenticide Impacts on Bobcats

N = 52



QUESTION: What gene pathways underlie increased disease susceptibility in bobcats exposed to anticoagulant rodenticides?

Sample Collection

- Not easy to carry out RNA-Seq on an existing sample set >> need to set out to collect RNA at the outset
- Gene expression is affected by so many factors that it is really important to have your study design clear ahead of time!
- RNA Is fragile- need specialty buffers (PaxGene, Tempus, RNALater) or extreme cold (liquid nitrogen) to preserve high quality RNA. **Be sure to read manufacturer guidelines for preservation!!!**
- When handling animals, be sure to minimize noise by using consistent procedures, minimizing handling time, taking note of everything!!!

RNA degrading enzymes are EVRYWHERE!!!

To prevent/reduce degradation of your RNA, always ensure:

1. You are working in a room dedicated to RNA

2. Use equipment (lab coats, pipettes, tips, etc) also designated only for RNA… keep these in the RNA room when not in use

3. Use RNase Away on all surfaces and equipment that will come into contact with your sample prior to starting your lab work and **in addition to standard decontamination protocols**

4. Work quickly but carefully!

RNA EXTRACTION

Different types of RNA

- Coding
  - mRNA: carried from the nucleus to the cytosol where it is translated into protein; the primary target of most RNASeq studies on wild populations; transcript abundance use as a proxy for gene expression

- Non-Coding
  - miRNA: short (~22 bp), single stranded RNA species involved in RNA silencing and post-transcriptional regulation of gene expression; present in plants, animals and some viruses

  - siRNA: small, double stranded RNA species that operated in tandem with Dicer protein to degrade messanger RNA, preventing translation into protein; also involved in anti-viral activity and chromatin shaping

Different protocols/kits will be necessary depending on the target RNA

RNA extraction kits

- The kit you choose will depend on preservation buffer and tissue type.
- Here is a list of the most popular

Extraction protocol	Preservation Buffer	Tissue Type	Link
RiboPure/RiboPure Blood *Phenol/chloroform	RNA*later*	Tissue or cultured cells/ whole blood	https://www.thermofisher.com/order/catalog/product/AM1924
PureLink/ PureLink Blood *guanidine-isocythionate	RNA*later*	Wide range of cell and tissue types/ whole blood	https://www.thermofisher.com/us/en/home/life-science/dna-rna-purification-analysis/rna-extraction/rna-types/total-rna-extraction/purelink-rna-mini-kit.html
Rneasy *guanidine-isocythionate	RNA*later*	Cells, tissues and yeast	https://www.qiagen.com/us/shop/sample-technologies/rna/rneasy-mini-kit/#orderinginformation
PaxGene	PaxGene	Tissue/ whole blood	http://www.preanalytix.com/products/blood/RNA/paxgene-blood-rna-tube
Trizol (or Trizol LS) *Phenol/chloroform	Trizol, RNA*later*	Wide range of cell and tissue/ whole blood	https://tools.thermofisher.com/content/sfs/manuals/trizol_reagent.pdf

Plant RNA extraction kits

Extraction Protocol	Link
Qiagen Plant RNeasy mini kit	https://www.qiagen.com/us/shop/sample-technologies/rna/rneasy-plant-mini-kit/#productdetails
Agilent	http://www.genomics.agilent.com/en/product.jsp?cid=AG-PT-158&tabId=AG-PR-1153&_requestid=290959
Sigma Aldrich Spectrum Plant Total RNA Kit	http://www.sigmaaldrich.com/life-science/molecular-biology/plant-biotechnology/plant-molecular-biology/product-highlights/spectrum-plant-total-rna-kit.html
MO BIO Power Plant Isolation Kit	https://mobio.com/products/rna-isolation/plant/powerplantr-rna-isolation-kit.html

Thoroughly read the kit specifications and current literature to decide which kit is appropriate for your project.

Bacterial RNA

Extraction Protocol	Kit
RNeasy Protect Bacteria Mini Kit	https://www.qiagen.com/us/shop/sample-technologies/rna/rneasy-protect-bacteria-mini-kit/#orderinginformation
Thermo Fisher Bacterial RNA Extraction	https://www.thermofisher.com/us/en/home/life-science/dna-rna-purification-analysis/rna-extraction/rna-sample-extraction/bacterial-rna-extraction.html
Sigma Aldrich Total RNA Bacteria Purification Kit	http://www.sigmaaldrich.com/life-science/molecular-biology/dna-and-rna-purification/total-rna-bacteria.html

Thoroughly read the kit specifications and current literature to decide which kit is appropriate for your project.

Preservation x Extraction for whole blood

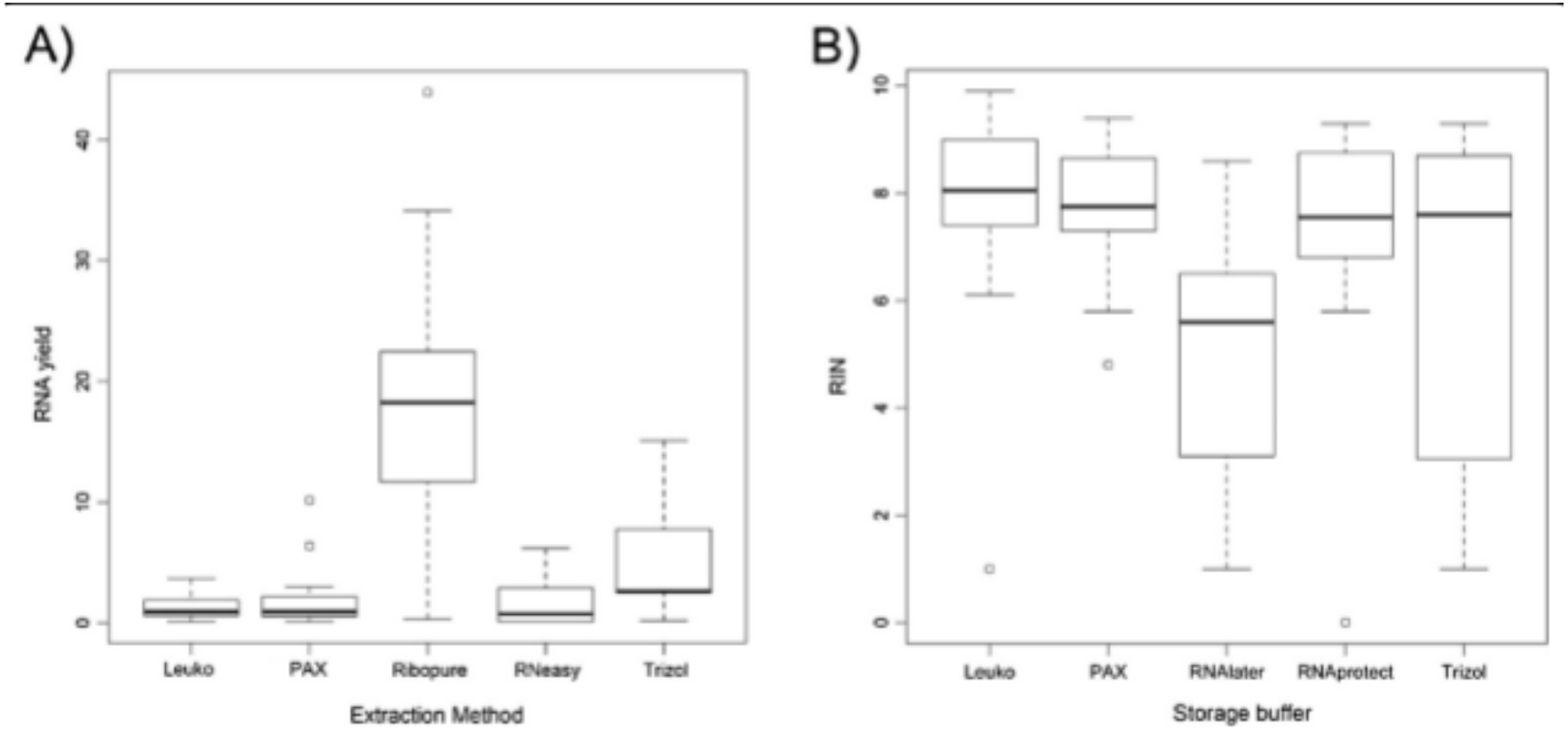
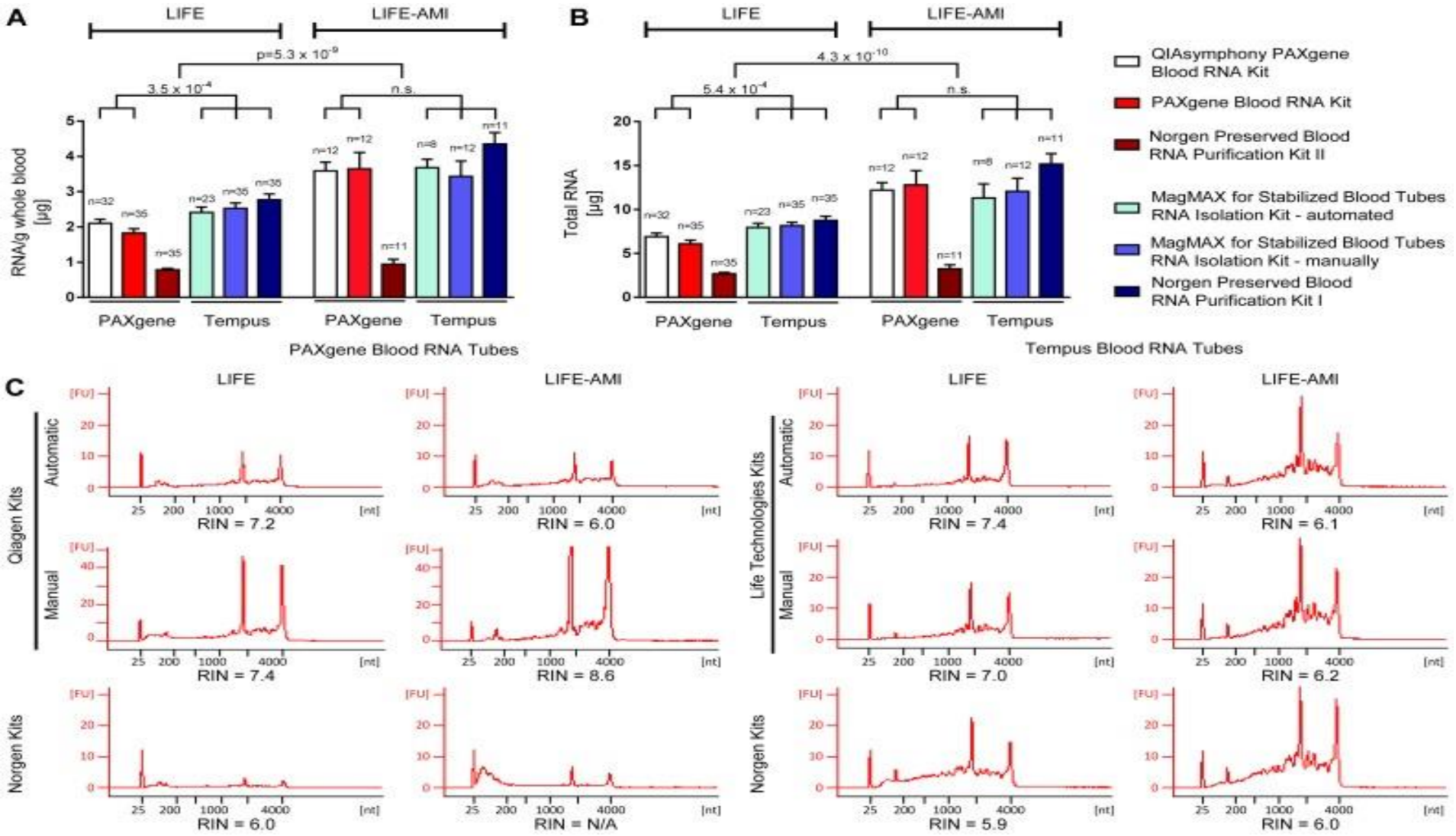


Figure 2 RNA yield and RIN values. RNA yield and RIN values for the five different RNA extraction protocols. (**A**) RNA yield (in µg) obtained after one or (if required) two DNase treatments from 500 µl blood using four different protocols/ kits for RNA extraction from whole blood as well as the LeukoLOCK™ filter system. (**B**) RIN values of the RNA extracted from blood samples preserved in different buffers. Please note that samples collected with the RNA filter "Leuko" were stored in RNA*later*® just as the whole blood samples "RNAlater". The RIN values of both collection methods differ dramatically and represent both the upper and lower limit of the performance of all tested preservation buffers.

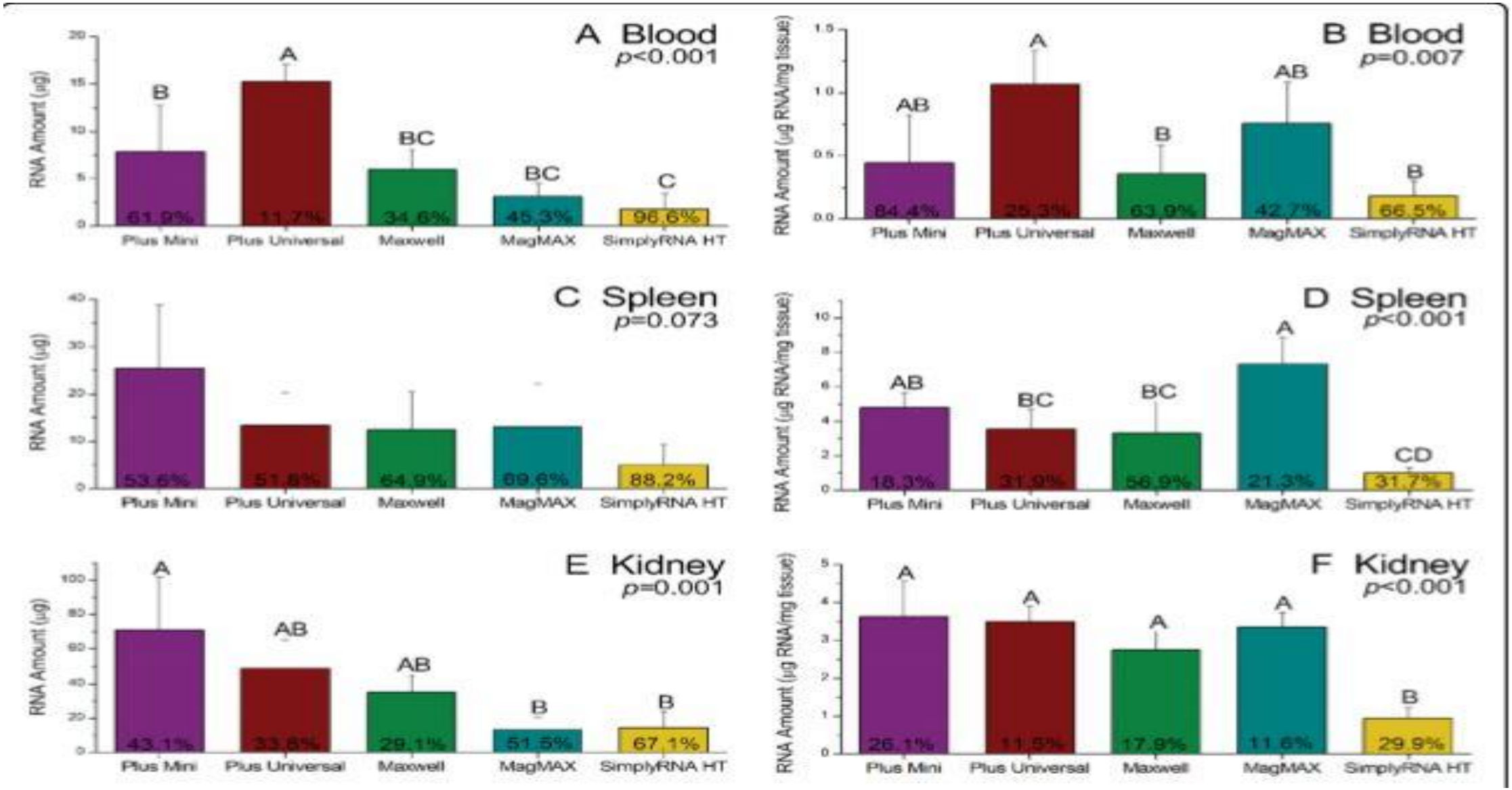
Schwochow et al. BMC Biotechnology 2012, 12:33

Preservation x Extraction for whole blood



Hantzsch M et al. PLoS ONE 2014; 9(12)

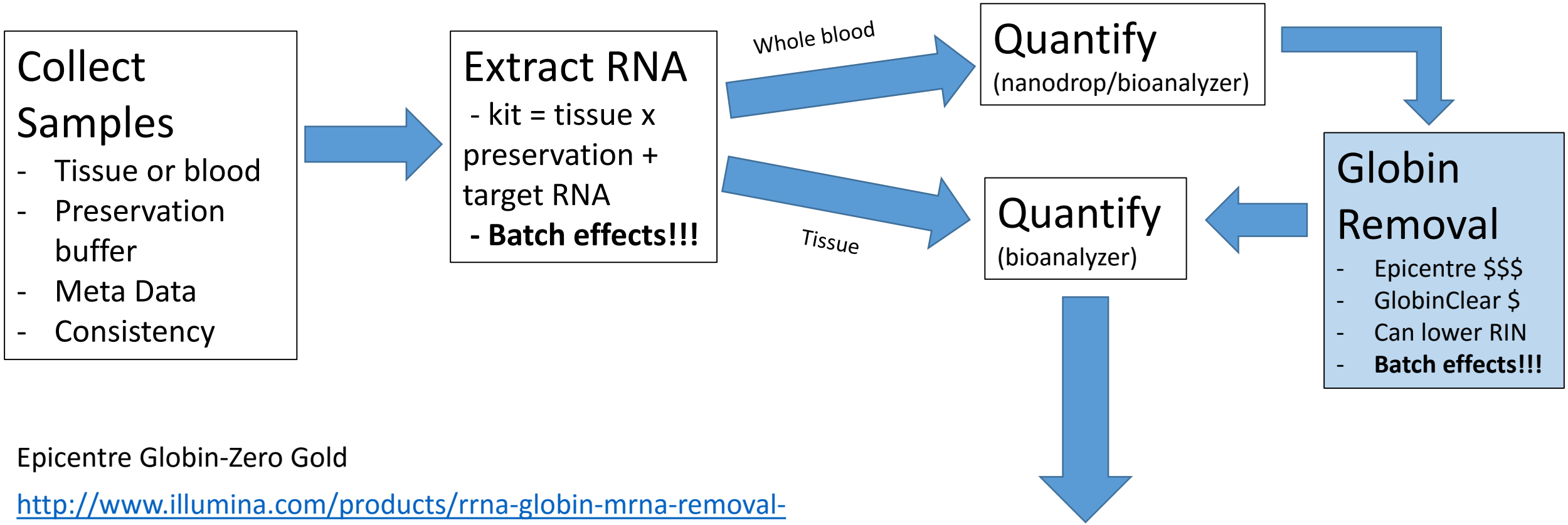
Comparison of Tissue x Extraction Kit



Sellin Jeffries et al. BMC Biotechnology 2014, 14:94

DNAse Treatment

- You want to remove any DNA that co-purified with your RNA using DNAse.
- Some protocols have this step built in, other have to be modified.
- Generally, you want to do just prior to the washing steps.
- An example from the Wayne Lab modified Trizol Extraction protocol (http://openwetware.org/wiki/Wayne:Laboratory_Protocols#Ambion_Trizol_Plus_RNA_purification_kit_protocol)
- 80 ul per sample:
  - 10 ul Purelink DNase
  - 8 ul Buffer
  - 62 ul H2o



Epicentre Globin-Zero Gold

http://www.illumina.com/products/rrna-globin-mrna-removal-kit-selection-guide.html

Ambion GlobinClear

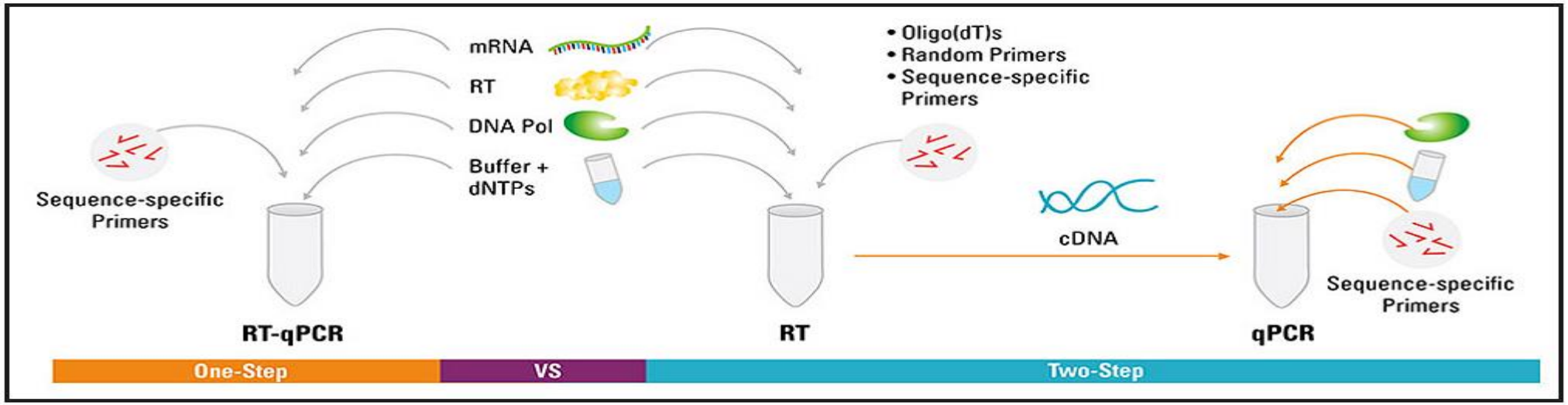
https://www.thermofisher.com/order/catalog/product/AM1981

RT-qPCR

1. One or two-step; both require reverse transcriptase
2. Sequence specific primer to detect expression of gene of interest
3. qPCR to amplify target RNA

→ Useful prior to initiating an RNA-Seq experiment to ensure a response can be detected (ie. Measure IL-8 following challenge to LPS)

→ Or following an RNA-Seq analysis once candidate genes have been identified and a more controlled experiment may follow



Library Preparation Considerations

- Sequencing platform
  - Illumina HiSeq
  - PacBio RS
  - Life Technologies PDM
  - Helicos
  - SOLiD
  - Roche 454
- Strand-specific (1st strand sequencing only or both)
- Ribosomal depletion or poly-A capture
- Coverage
- Read lengths
- Cost

Sequencing Platforms

Table 2: Comparison of different sequencing technologies

Parameters \ Sequencing Technologies	Ion Torrent (Ion semi-conductor)	Roche 454 (Pyrosequencing)	Illumina (Sequencing by synthesis)	SOLiD (Sequencing by ligation)	Pacific Bio (Single molecule real-time sequencing)	Helicos (True Single Molecule Sequencing)	Sanger method (Chain termination)
Sequencing chemistry	Detection of released H^+	Pyrosequencing	Reversible terminators	Ligation	Fluorescently labelled dNTPs	Reversible terminators	Di- deoxy Chain termination
Adapter used	Adapters	Adapters	Adapters	Adapters	Hairpin adapters	Poly(A) adapter	N/A
Amplification method	Emulsion PCR	Emulsion PCR	Bridge amplification in situ	Emulsion PCR	Linear amplification	No amplification	Sequencing PCR
Separation method	Ion Spheres and high density array	Microbeads and 'picotitre' plate	Glass slide hybridization	Beads on glass slide	Captured by DNA polymerase in microcell	Flow-cell hybridization	Electrophoresis
Read length	200 -400 bp	700 bp	50 to 250 bp	50-75 bp	1000 bp	25 bp	400 to 900 bp
Reads per run	up to 5 million	1 million	up to 3 billion	1.2 to 1.4 billion	35-75 thousand	1 billion	Not available
Maximum data output per run	1 Gb	700 Mb	600 Gb	20 Gb	Not available	35 Gb	Not available
Accuracy	98%	99.9%	98%	99.9%	99%	99%	99.9%
Time per run	2 hours	24 hours	1 to 10 days	1 to 2 weeks	30 minutes to 2 hours	5-10 days	20 minutes to 3 hours
Cost per 1 million bases (in US$)	$1	$10	$0.05 to $0.15	$0.13	$2	Not available	$2400
Advantages	• Equipment relatively less expensive • Fast reaction	• Long read size • Fast reaction	• High sequence yield	• Low cost per base of sequencing	• Longest read length • Less time consuming	• No PCR induced bias and errors • Tolerates degraded samples	• Long individual reads. • Applied in many sequence based research
Disadvantages	• Homopolymer error	• Homopolymer error • Runs relatively expensive	• Needs high DNA concentration • Very expensive equipment.	• Slower than other sequencing methods.	• Low yield at high accuracy • Equipment very expensive.	• Time to sequence a single nucleotide is high • High error rate	• Higher cost per base of sequencing • Impractical for whole genome sequencing projects

Minakshi et al. Advances in Animal and Veterinary Sciences 2014: 2 (4S): 55 – 63

RNA library kits

Kit	Link
TruSeq	http://www.illumina.com/products/truseq_rna_library_prep_kit_v2.html
Kapa Biosystems	https://www.kapabiosystems.com/product-applications/products/next-generation-sequencing-2/rna-library-preparation-2/
Encore complete	http://www.nugen.com/products/ngs/encore-complete-rna-seq-library-systems
Ovation RNA Seq V2	http://www.nugen.com/products/ngs/ovation-rna-seq-system-v2
SMARTer stranded	http://www.clontech.com/US/Products/cDNA_Synthesis_and_Library_Construction/Next_Gen_Sequencing_Kits/Total_RNA-Seq/Strand-Specific_RNA_Seq_Illumina

Wayne Lab has used the TruSeq and Kapa kits.

Whichever you choose, use it throughout the study.

Adapters not included in the Kapa kit

Comparison of Kits

Table 1 Summary of the principal characteristics of the RNA-Seq library preparation kits evaluated in this study

	TruSeq stranded	Encore complete	Ovation RNA-Seq V2	SMARTer stranded
RNA input (minimal requirement according to the manufacturer)	100 ng depleted RNA	100 ng total RNA	0,5 ng depleted RNA	1 ng depleted RNA
Minimum quality	FFPE	RIN >7	FFPE	FFPE
rRNA depletion required	Yes	No	Yes	Yes
cDNA synthesis	Random primers	Selective priming	Random and oligo(dT) primers	Random primers
Fragmentation method	RNA by divalent cations + heat	cDNA by Covaris shearing	cDNA by Covaris shearing	RNA by heat
Strand selection	Yes	Yes	No	Yes
Library preparation method and reagents	Included	Included	Not included	Included
Multiplex capacity	96-plex	16-plex	according to the library preparation method chosen	12-plex
Experiment duration	6 hours	7 hours	4.5 hours for cDNA synthesis + time for library preparation	4.5 hours

FFPE: Formalin-fixed, paraffin-embedded tissue.
RIN: RNA integrity number.

**Kapa Biosystems
100 ng

RIN > 7
No
?

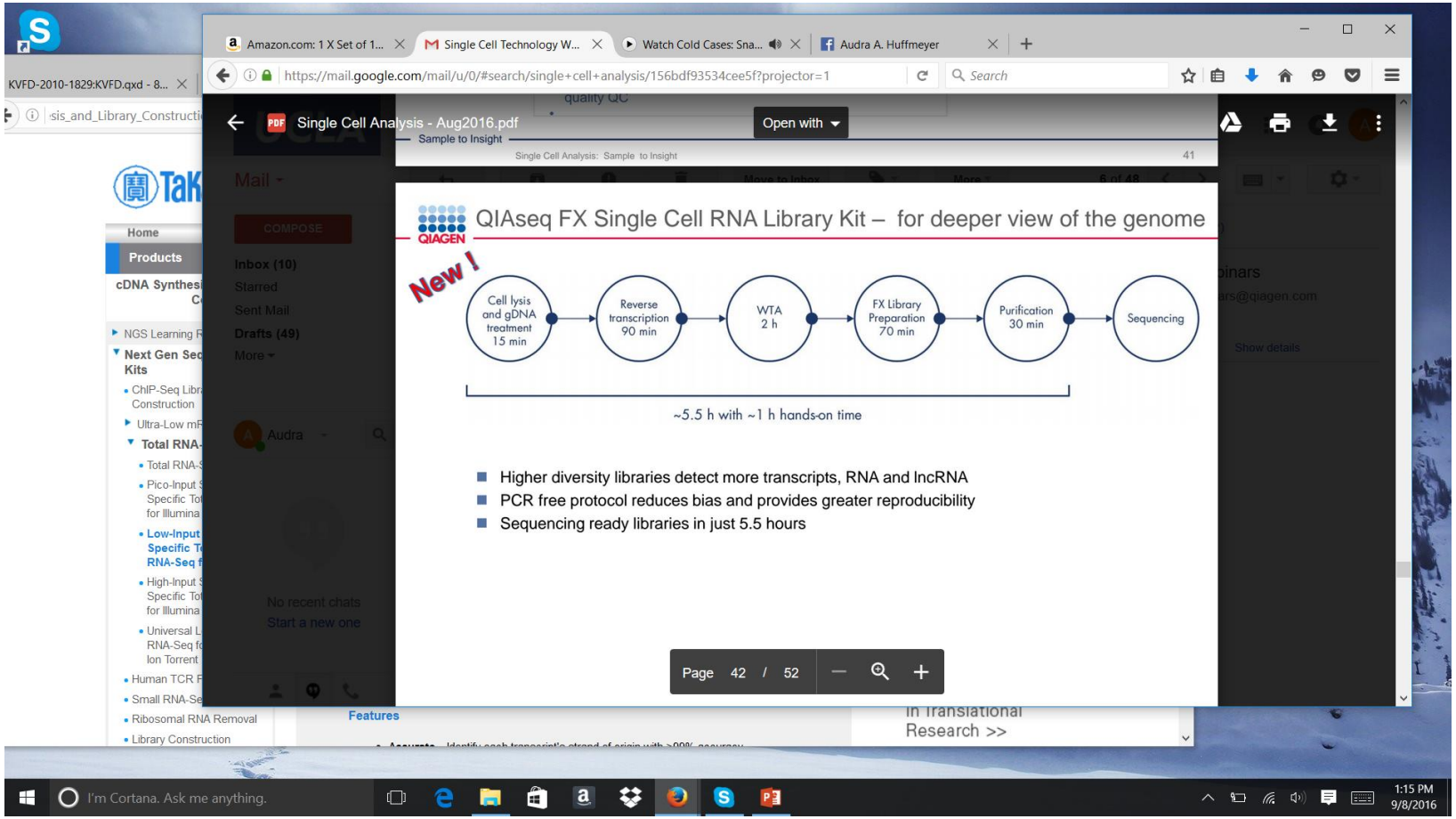
RNA by heat
Yes
Included

Adapters not included; compatible w/ TruSeq

8+ hours

Modified from Alberti et al. BMC Genomics 2014, 15:912

Single Cell, RNA to Library!



Qiagen