RNA sequencing : library preparation and experimental design

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RNA sequencing

- Introduction
- Preparation of RNA-seq libraries
- Design of RNA-seq experiments
- RNA-seq bias already identified

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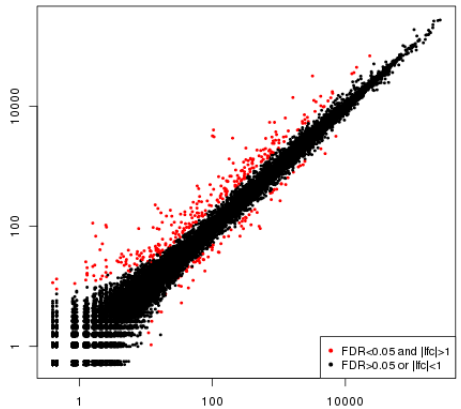
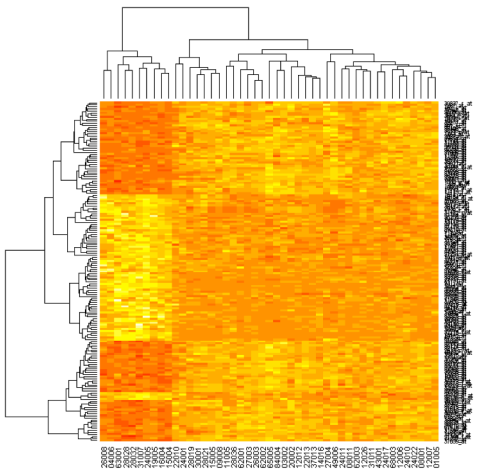
Transcriptome analysis : key aims

- Quantitative
  - Quantify the changes of expression level between different conditions / time points

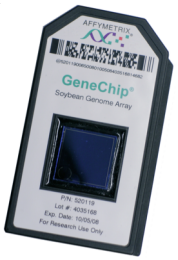




- Qualitative
  - Catalogue all different transcripts (mRNA, ncRNA)
  - Determine the structure of these transcripts
    - TSS, 3’ end, splicing patterns, post-transcriptional modifications

Transcriptome analysis : different technologies

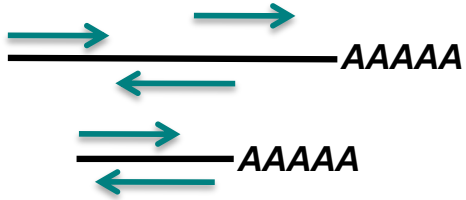
- Hybridization-based approach
  - Microarrays



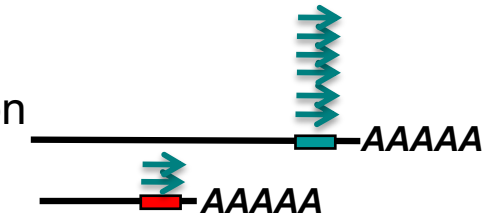
  - Drawbacks
    - Reliance upon existing knowledge on transcriptome
    - Poor quantification of lowly (background) and highly (saturation) expressed genes
    - Cross-hybridization

Transcriptome analysis : different technologies

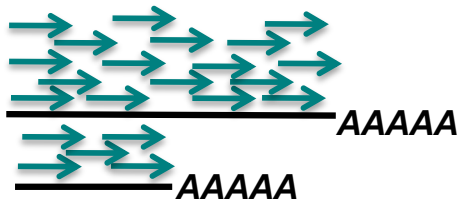
- Sequence-based approaches
  - EST (Expressed Sequence Tag)
    - Sequence of a cDNA fragment
    - Drawbacks
      - Sanger sequencing → low throughput
      - Generally not quantitative (normalized libraries)



  - SAGE (Serial Analysis of Gene Expression)
    - Sequence a tag : short fragment from a specific location of each transcript
    - Drawback : only a portion of the transcript is analysed (isoforms are generally indistinguishable from each other)



  - RNA-seq
    - Sequence cDNA fragments from the whole cDNA
    - Qualitative and quantitative information



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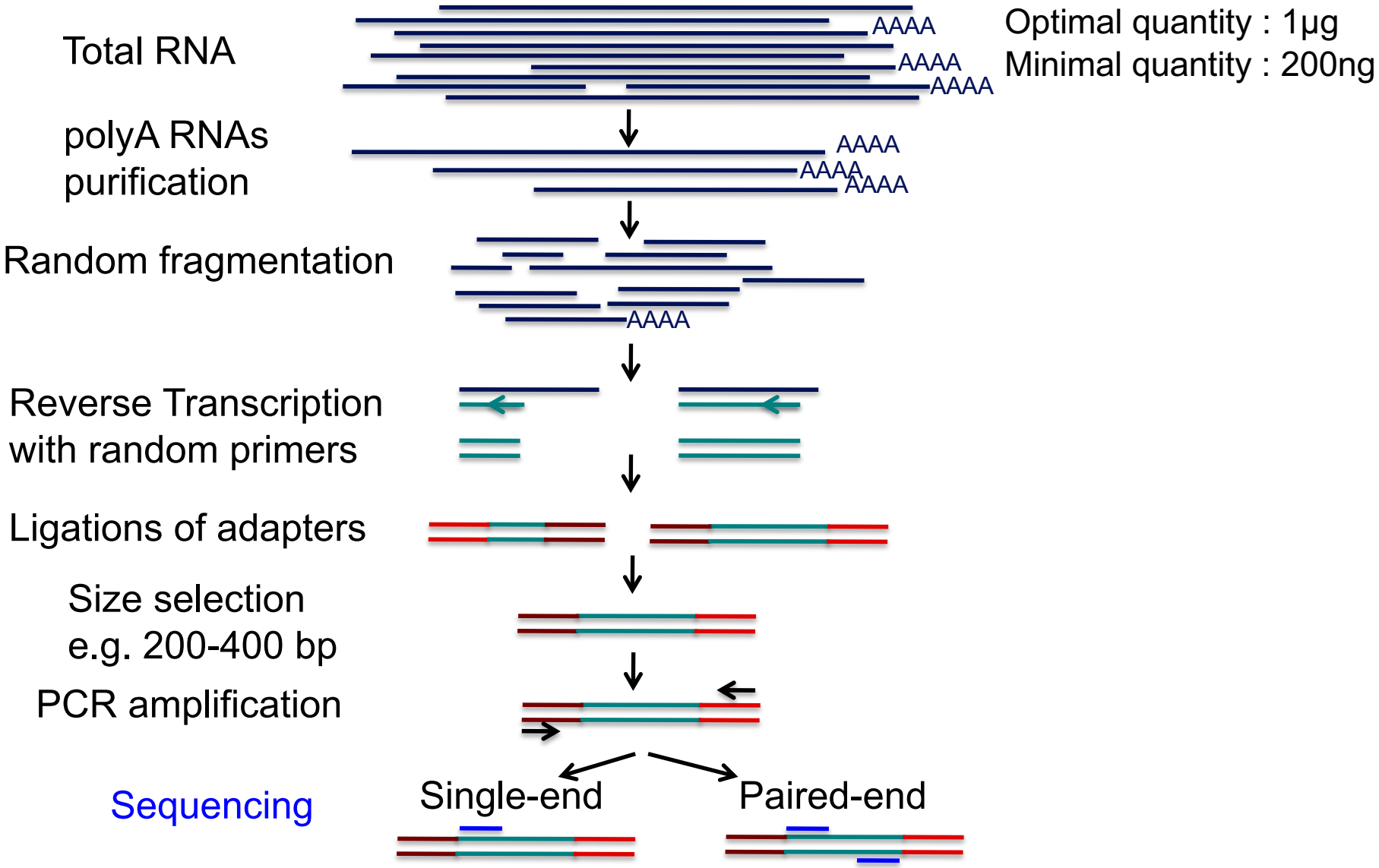
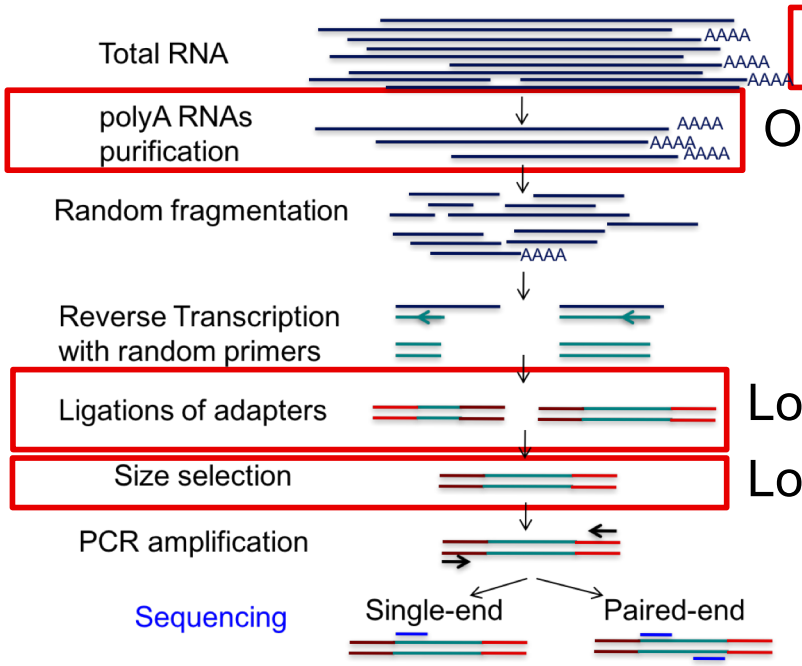
RNA-seq library preparation



RNA-seq library preparation

- Advantages
  - Highly reproducible
  - High sensitivity
  - Allows to study both coding and non-coding polyA+ RNAs expression
  - Allows transcript discovery
- Limitations



Optimal quantity : 1µg
Minimal quantity : 200ng

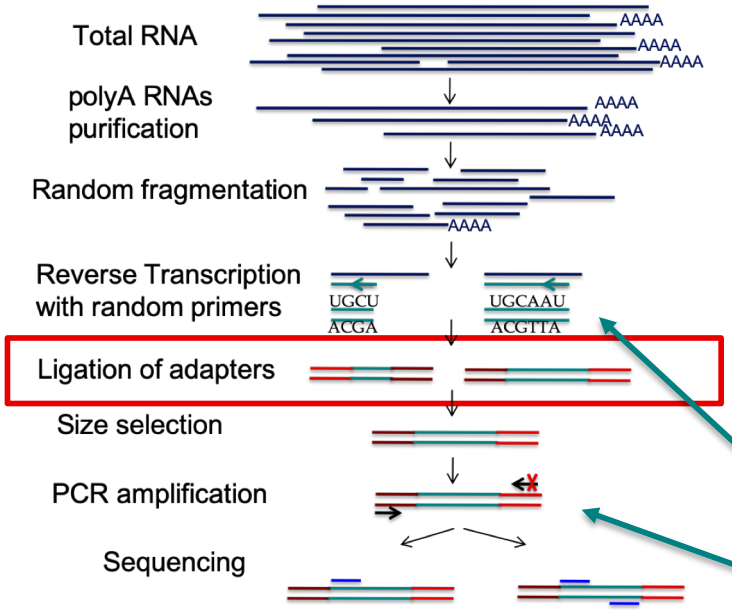
Quantity of starting material

Only polyadenylated RNA are studied

Loss of strand information

Loss of small RNAs (~ < 100 bp)

RNA-seq library preparation : stranded protocols



Loss of strand information

Stranded (directional) protocols

Incorporation of dUTP instead of dTTP in the second strand cDNA synthesis

Amplification of reverse strand only (high fidelity Taq polymerase)

PolyA+ RNA
→ Illumina TruSeq Stranded mRNA-Seq

Not limited to polyA+ RNA
→ Illumina TruSeq Stranded Total RNA-Seq

- Advantages
  - Preserves the strand information
    - ➔ Allows to determine transcript orientation
    - ➔ Important for novel transcript discovery and annotation, especially for overlapping transcripts

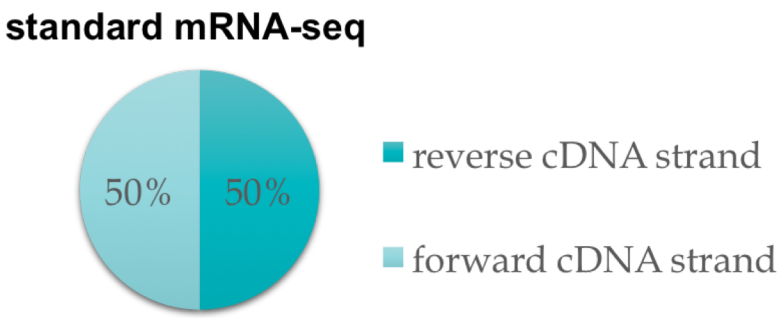
Optimal quantity : 1 µg
Minimal quantity : 200 ng

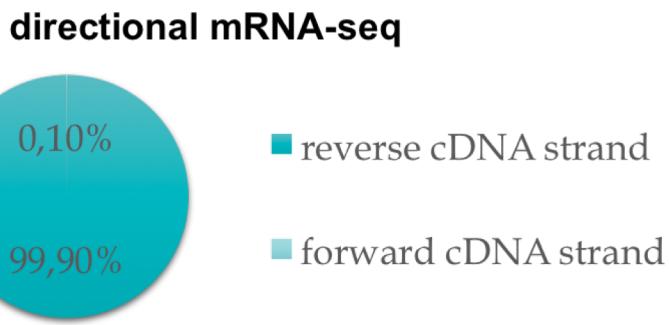
RNA-seq library preparation : stranded protocols

- Good quality of strand-specificity

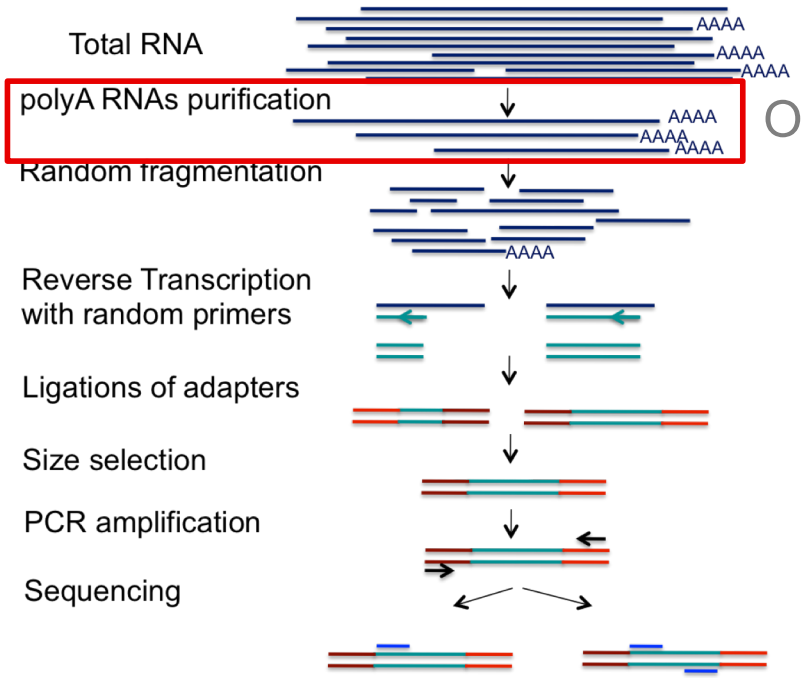
  e.g. Results obtained on spike-in RNAs added in 4 libraries prepared with both standard and directional polyA+ RNA-seq protocols *(GenomEast Platform)*

  Proportion of reads from each cDNA strand :





RNA-seq library preparation : protocols not limited to polyA+ RNA



Only polyadenylated RNA are studied

Protocols not limited to polyA+ RNA :

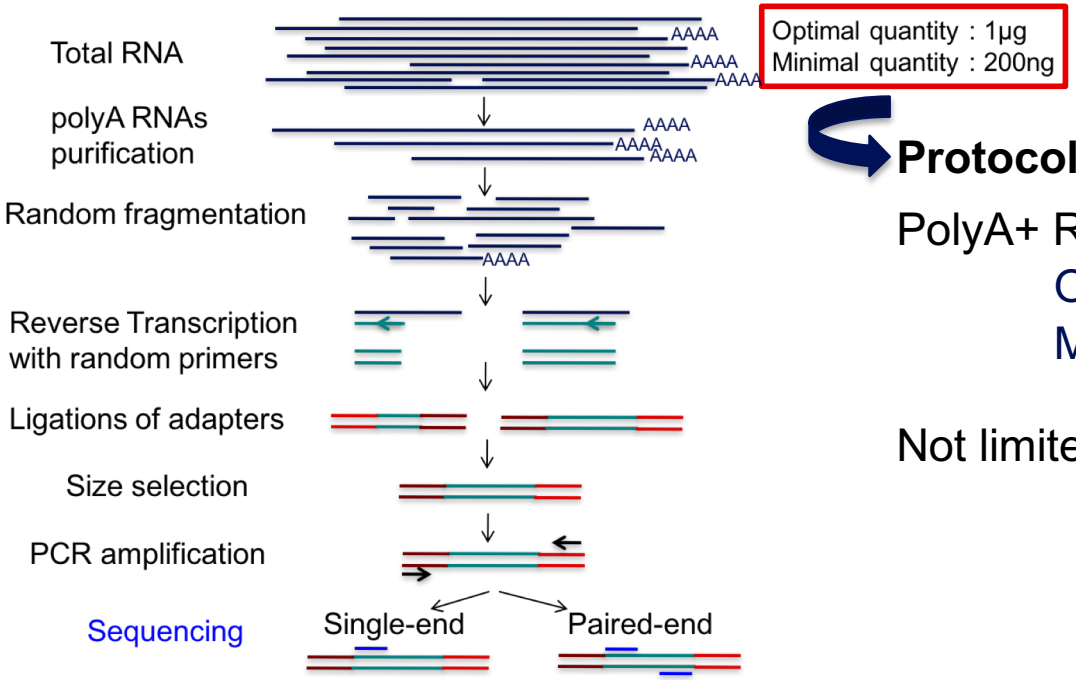
With amplification
→ NuGEN Ovation RNA-seq
(RT primers specific to non rRNA sequences)
Optimal quantity : 10 ng
Minimal quantity : 500 pg

Without amplification
→ Illumina TruSeq Stranded Total RNA-seq
(RiboZero probes used for rRNA depletion)
Optimal quantity : 1 µg
Minimal quantity : 100 ng

- Advantage
  - Allows to study non-polyadenylated transcripts
- Drawbacks
  - Efficiency of rRNA removal ≠ between samples
  - Higher number of RNA molecules sequenced compared to standard RNA-seq

➔ More reads needed to achieve the same coverage on polyadenylated RNAs

RNA-seq library preparation : protocols with amplification



Optimal quantity : 1µg
Minimal quantity : 200ng

Quantity of starting material

Protocols with amplification :

PolyA+ RNA → Clontech SMART-Seq

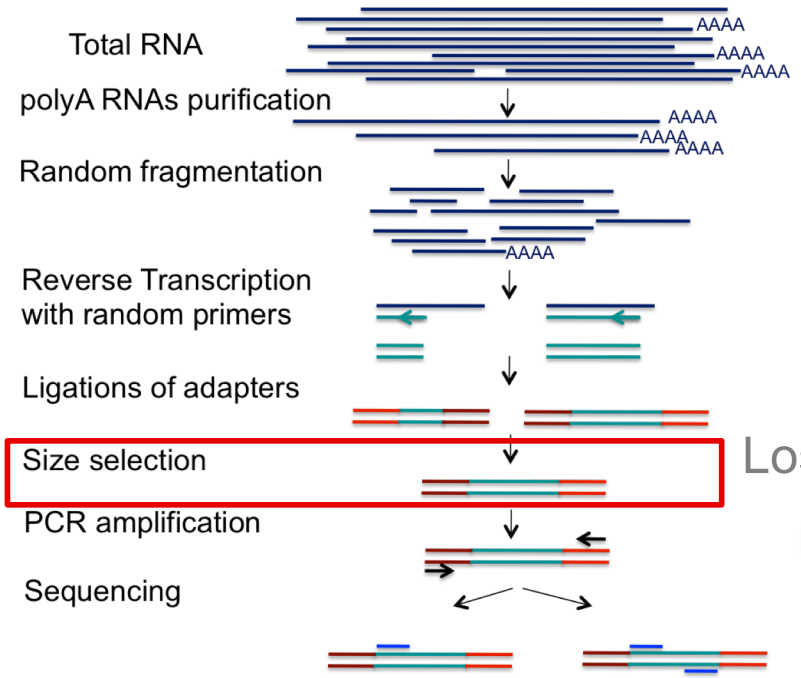
Optimal quantity : 10 ng
Minimal quantity : 100 pg or 100 cells

Not limited to polyA+ RNA → NuGEN Ovation

Optimal quantity : 10 ng
Minimal quantity : 500 pg

- Advantage
  - Low quantity of starting material
- Drawback
  - Bias due to the amplification

small RNA-seq library preparation



Loss of small RNAs (~ < 100 bp)

Small RNA-seq protocol

small RNA-seq library preparation

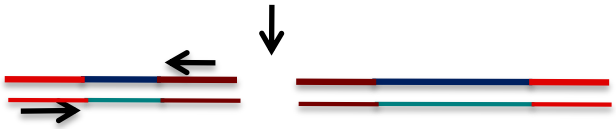
Illumina Truseq smallRNA SamplePrep

Optimal quantity : 2µg
Minimal quantity : 1µg

Total RNA

Ligation of adapters

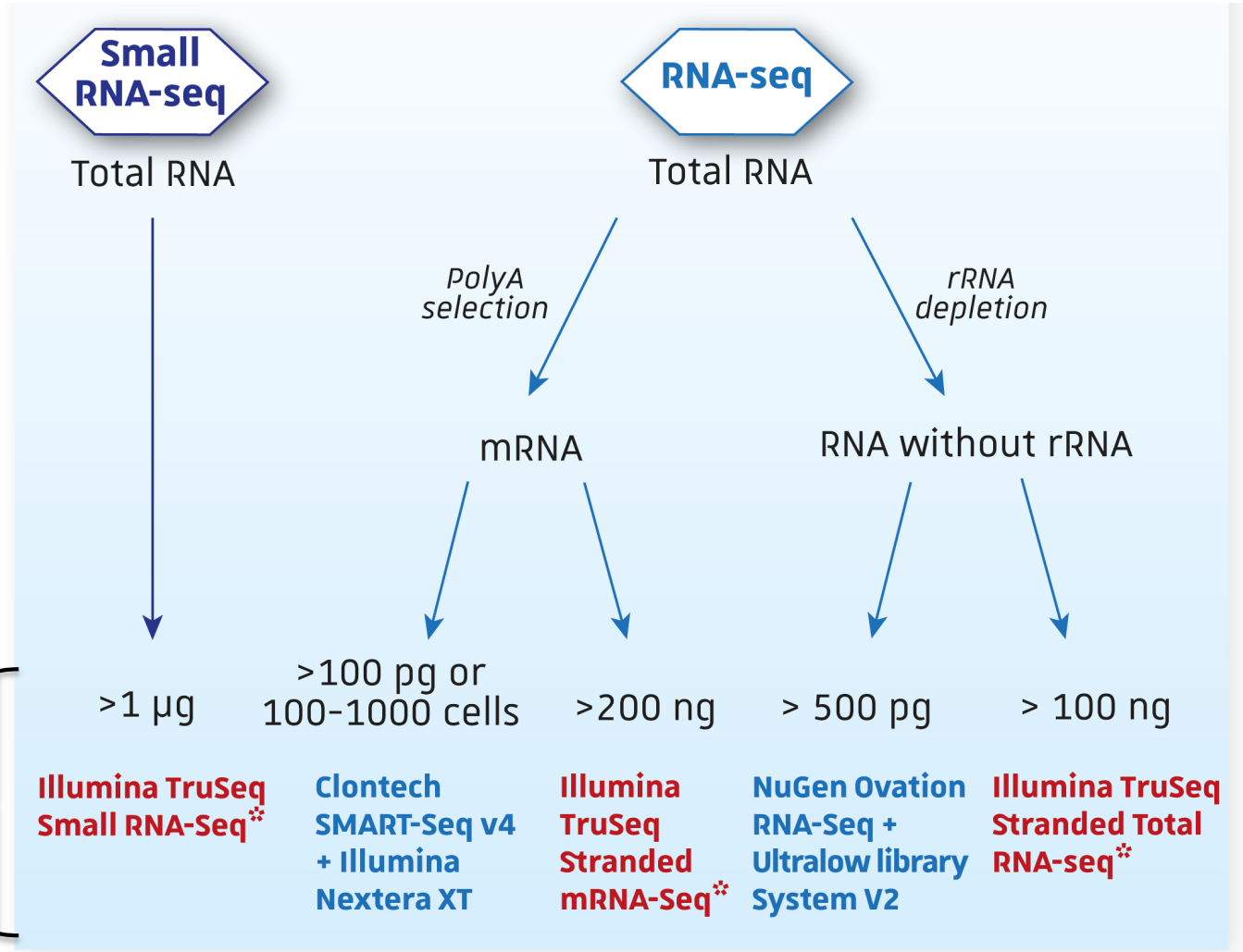
RT 1st strand synthesis
PCR amplification



Adapted size selection

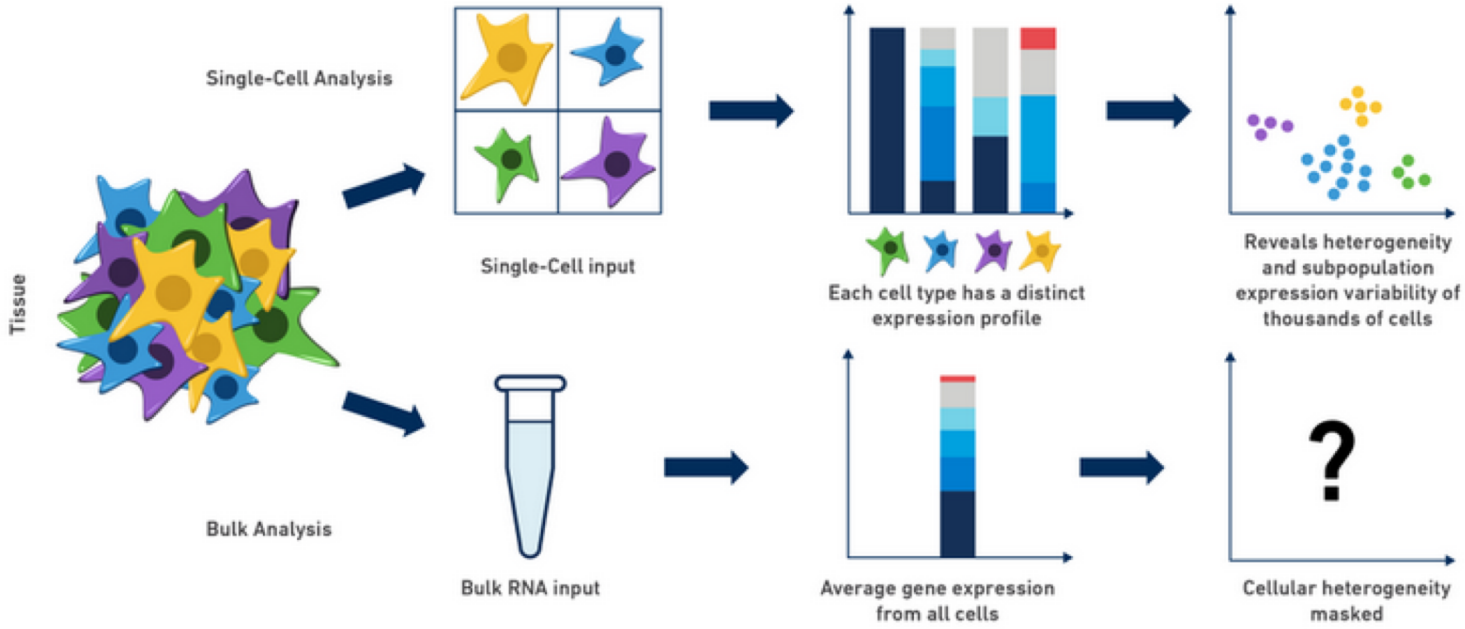
Sequencing

RNA-seq library preparation protocols (bulk)



Single-cell RNA-seq

- Majority of RNA-seq experiments : study of a cell population
- Overlooks differences within a cell population that may be important for maintaining normal tissue function or facilitating disease progression
- Single-cell RNA-seq provides the expression profiles of individual cells
  - Allows to characterize the subpopulation structure
  - Allows to study cell heterogeneity



From community.10xgenomics.com

Single-cell RNA-seq

- Different technologies for single cell collection
  - Droplets (e.g. Chromium, 10X Genomics)
  - Microfluidics (e.g. C1 single-cell auto-prep system, Fluidigm)
  - Microwells (e.g. Rhapsody single-cell analysis System, BD)
- Different protocols for RNA-seq
  - 3’ counting or full-length
  - With or without Unique Molecular Identifiers (UMI)
    - Random sequences used to tag each molecule prior to library amplification
    - 2 reads align to the same location and have the same UMI
      → highly likely PCR duplicates
- Limits
  - Technical noise due to amplification and dropout

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Experimental design

1. Define your biological questions of interest
2. Define the best appropriate experimental design to answer these questions :
   - Library preparation protocol
   - Sequencing strategy
   - Number of reads
   - Number of replicates

- Define a detailed experimental plan in advance of doing the experiment
- Try to reduce batch effects

- ENCODE guidelines (mammalian tissues)

  https://www.encodeproject.org/about/experiment-guidelines/

Which protocol for which application ?

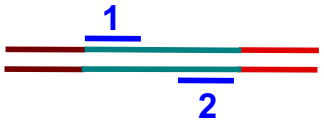
- Choice depend on
  - Quantity of starting material
  - Type of RNA studied (small/long, polyA+/-)
  - Biological questions of interest
    - e.g. new transcript identification → directional protocol

- Keep the same protocol for all samples within a project

Which protocol for which application ?

Library preparation	Kit used by the platform	Total RNA quantity		Type of studied RNA	Stranded
		Minimal	Optimal		
mRNAseq/ standard quantity	TruSeq RNA Sample Prep	200 ng	1µg	Only polyA+ RNA of size > 100 b	No
Stranded mRNAseq/ standard quantity	Directional mRNA-Seq SamplePrep	200 ng	1µg	Only polyA+ RNA of size > 100 b	Yes
mRNAseq/low input (Smarter)	SMART-Seq v4 UltraLow Input RNA kit + Nextera XT DNA sample preparation Kit	100 cells	10 ng	Only polyA+ RNA of size > 100 b	No
mRNA-seq/ single cell	SMARTer Ultra Low RNA Kit for the Fluidigm C1 System + Nextera XT DNA sample preparation Kit	1 cell	1 cell	Only polyA+ RNA of size > 100 b	No
Total RNAseq Ribozero/standard quantity	Truseq Stranded Total RNA SamplePrep	100 ng	1 µg	All RNA of size > 100 b	Yes
Total RNAseq/ low input (Ovation)	Ovation RNA-Seq System V2 + Ovation SP Ultralow Library systems	500 pg	10 ng	All RNA of size > 100 b	No
Small RNA-seq	Truseq SmallRNA SamplePrep	1 µg	2 µg	All small RNAs with 5'P and 3'OH (desired size can be chosen by the project manager)	Yes

Which sequencing strategy ?

- Expression quantification on annotated transcripts
  - Single-end sequencing provides good results 
- Alternative splicing analysis, fusion transcript detection, mapping over repetitive regions, de novo transcriptome assembly
  - Paired-end sequencing is needed 

How many reads are needed ?

- Transcriptome coverage as a function of sequencing depth: highly dependant on transcriptome complexity
- Sequencing depth should be determined by the goals of the experiment
- General recommendations for typical mammalian tissues
  - > 30 million reads with polyA+ protocols
  - > 50 million reads with total protocols
  - … if the goal is to quantify expression of annotated genes
- Higher sequencing depth needed if
  - the sensitivity of detection is important
  - the purpose is to discover novel transcripts
  - the purpose is to precisely quantify transcript isoforms

Examples
on our Hiseq4000

Application	Suggested multiplexing *for standard experiments on mammalian genomes*
small RNA-seq	20 samples / lane
mRNA-seq with polyA selection	
→ *for gene expression quantification*	8 samples / lane
→ *for alternative splicing analysis*	3 samples / lane
RNA-seq with ribodepletion	
→ *for gene expression quantification*	5 samples / lane

How many replicates are needed ?

- Low technical variability
  and technical variability << biological variability
  (Marioni et al. Genome Research 2008. Bullard et al. BMC Bioinformatics 2010)
  ➔ Technical replicates not required
- But “sequencing technology does not eliminate biological variability”
  (Hansen et al. Nat Biotechnol. 2011)
  - **Biological replicates are fundamental !**
  - How many ?
    - Highly dependant on the correlation between replicates and on the difference between the compared conditions
    - If possible, prepare more samples for low-input RNA-seq

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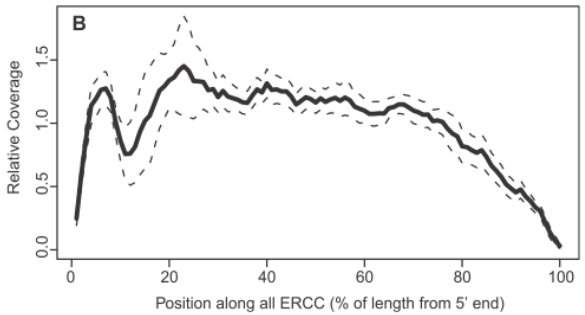
RNA-seq bias / sources of variability

- As all techniques, RNA-seq present bias affecting expression estimates and subsequent statistical analysis

- Identification of bias in RNA-seq protocol
  - Use of synthetic spike-in standards
    (Jiang et al. Genome Research 2011;21(9):1543-51)
  - Provided by ERCC (External RNA Control Consortium)
  - 92 sequences
  - Minimal sequence homology with endogenous transcripts from sequenced eukaryotes
  - Various lengths and GC content, large range of concentrations

RNA-seq bias / sources of variability

- Composition bias of the first 13 nucleotides due to a non-random hexamer priming
  (Hansen et al. 2010;38(12):e131. Li et al. Genome Biology 2010;11(5):R50)
- Bias during library amplification (Kozarewa et al. 2009;6(4):291-5)
  - Over-amplification of GC-rich regions
  - Generation of duplicate sequences
- Read coverage bias (Jiang et al. Genome Research 2011;21(9):1543-51)
  - Unevenness in read coverage along transcripts



- Variability in RNA-seq data (Marioni et al. Genome Research 2008;18(9):1509-17. Bullard et al. BMC Bioinformatics 2010;11:94)
  - Biological condition >> library preparation > run > lane

RNA-seq bias / sources of variability

- Transcript abundance
  - Low abundance transcripts more affected by sampling error : more bias in the estimation of their expression level
  - Highly dependant on the sequencing depth :
    - A question of cost, not due to the technique
- Transcript length (Oshlack et al. Biology Direct 2009;4:14)
  - The ability to call differentially expressed genes between samples is associated with the length of the transcript :

    more statistical power to detect differential expression for long transcripts compared to short ones
- Mappability bias
  - Uniquely mapping reads are typically summarized over genomic regions ➔ regions with lower sequence complexity will tend to end up with lower sequence coverage
  - Reads corresponding to longer transcripts have a higher mappability