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Central European Institute of Technology

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UNIVERSITAS MASARYKIANA BRUNENSIS

Moderní metody pro analýzu genomu: Bioinformatika I

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29. October 2018

EUROPEAN UNION
EUROPEAN REGIONAL DEVELOPMENT FUND
INVESTING IN YOUR FUTURE

2007–13
OP Research and Development for Innovation

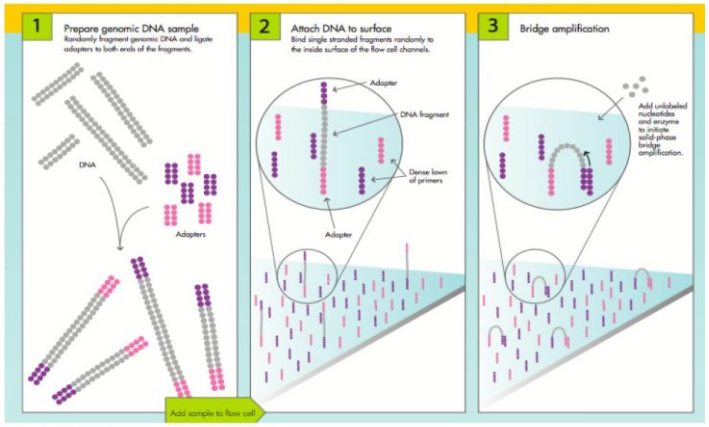
Goals of the presentation

- **Overview of NGS bioinformatics**
  - **NGS bioinformatics < Sequence analysis < Bioinformatics**
- **What to think about when you**
  - **plan experiment**
  - **discuss data analyses**
  - **check results**

- **Not to teach you how to do bioinformatics**

NGS Bioinformatics

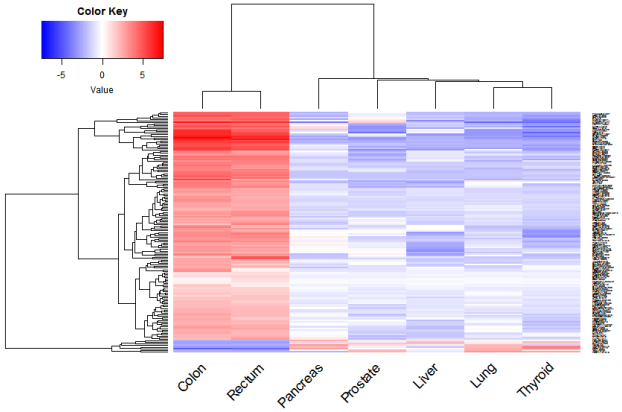
Illumina 1

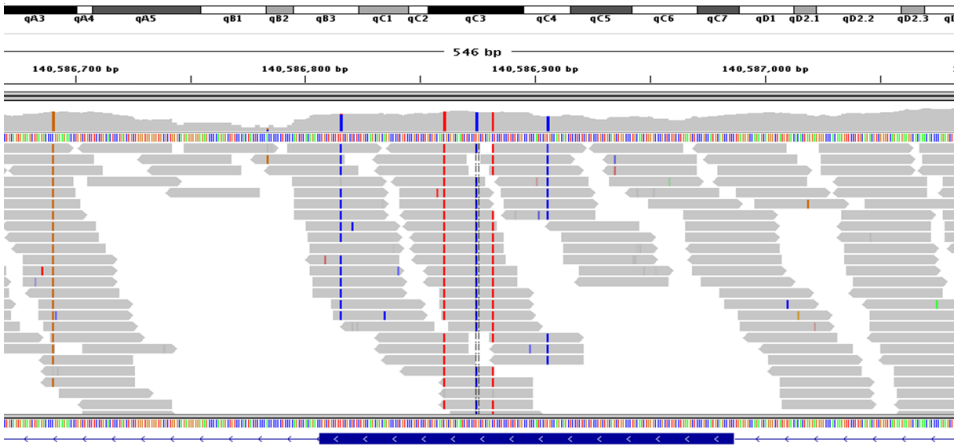


ECE/BioE 416
Lecture 24

10

Your raw sequence data





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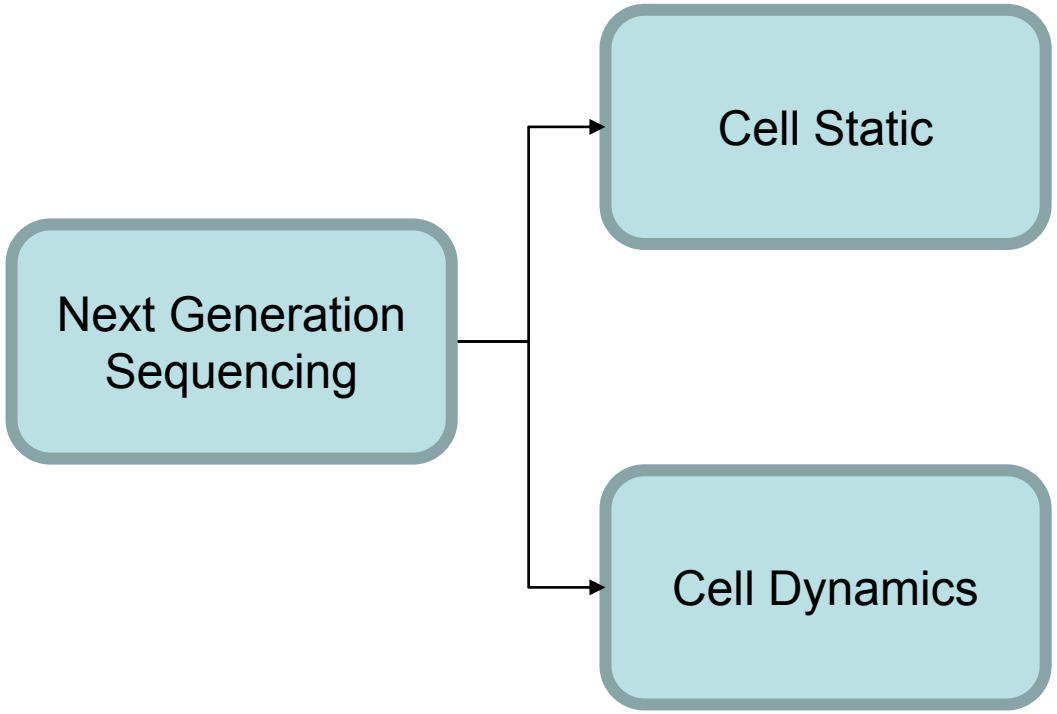
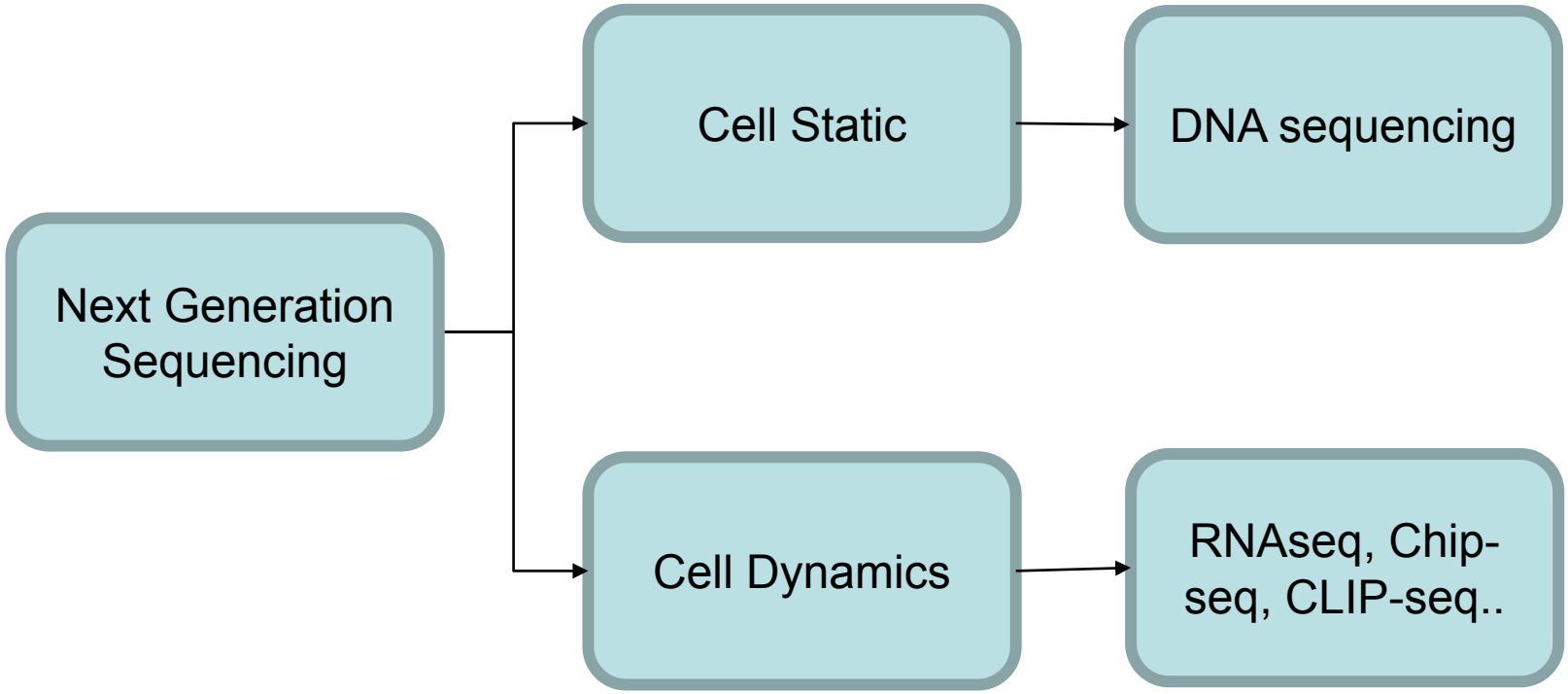
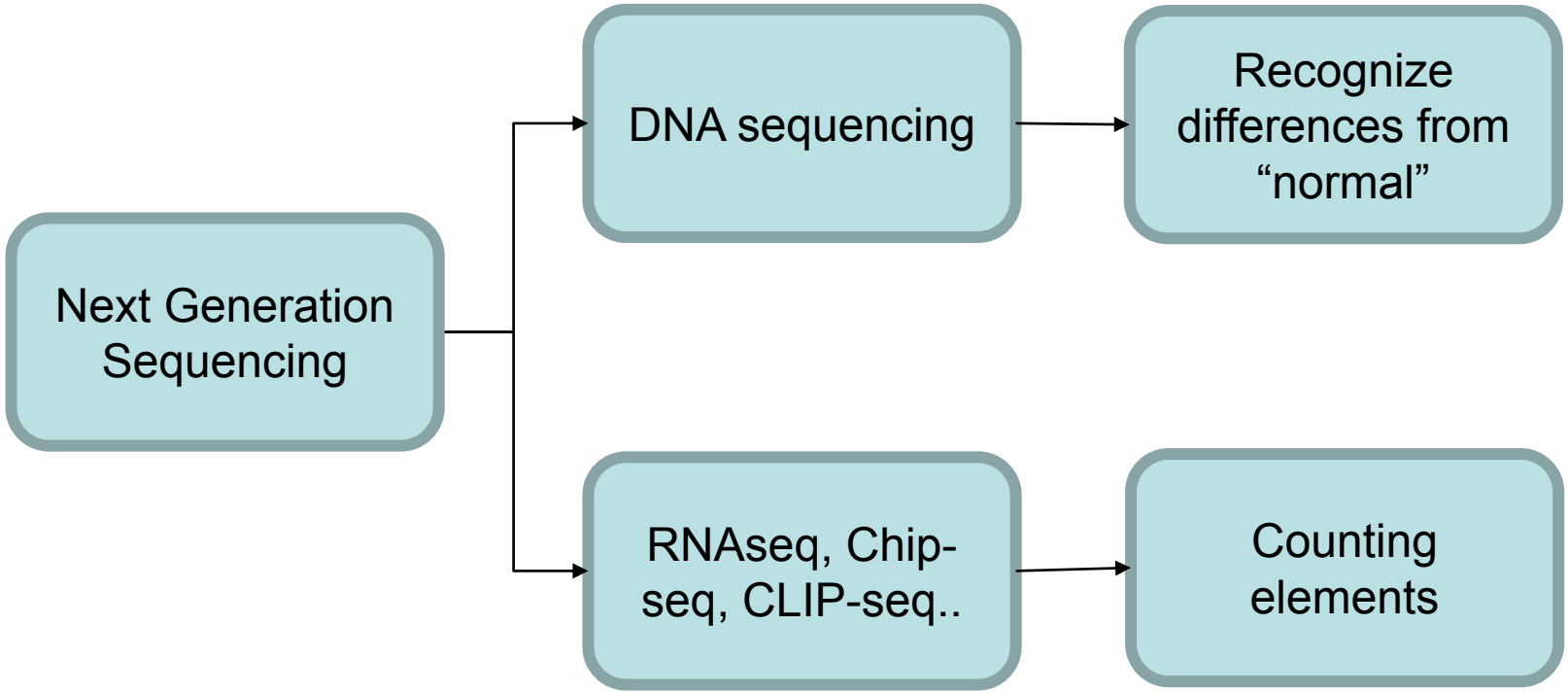
NGS experiments



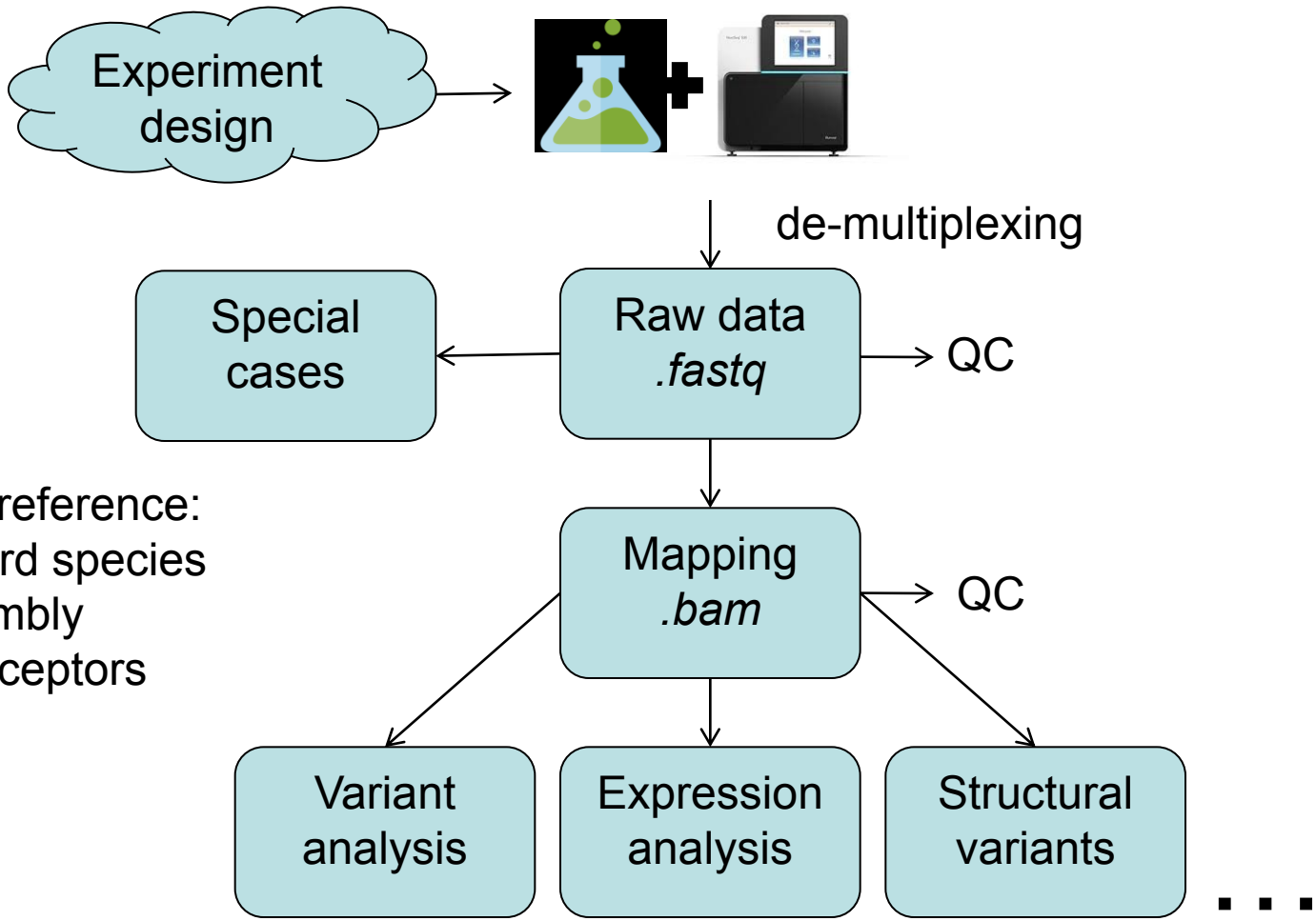
NGS experiments



NGS experiments

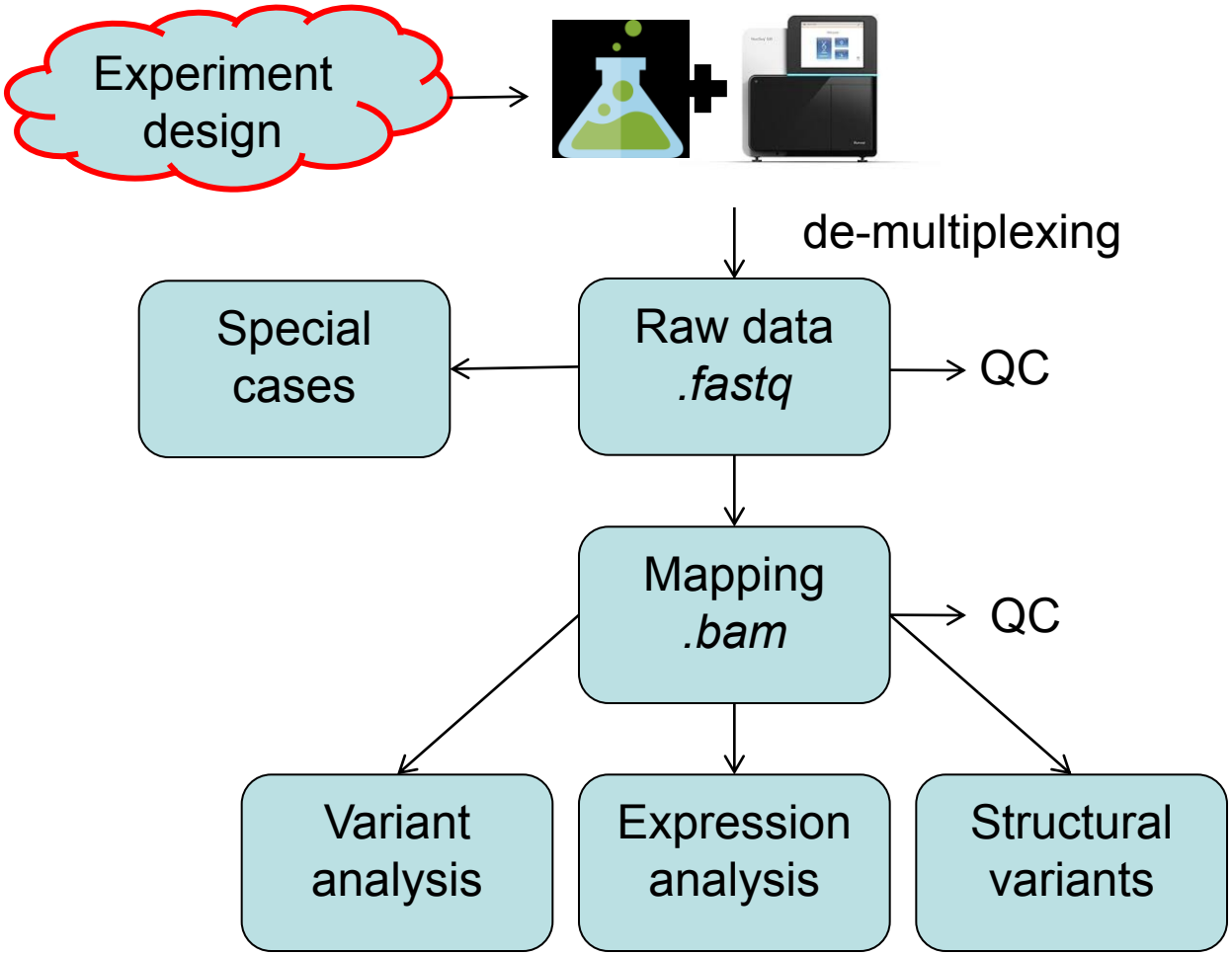


NGS data analysis workflow



Not known reference:
Non-standard species
Assembly
IG/TR receptors

NGS data analysis workflow

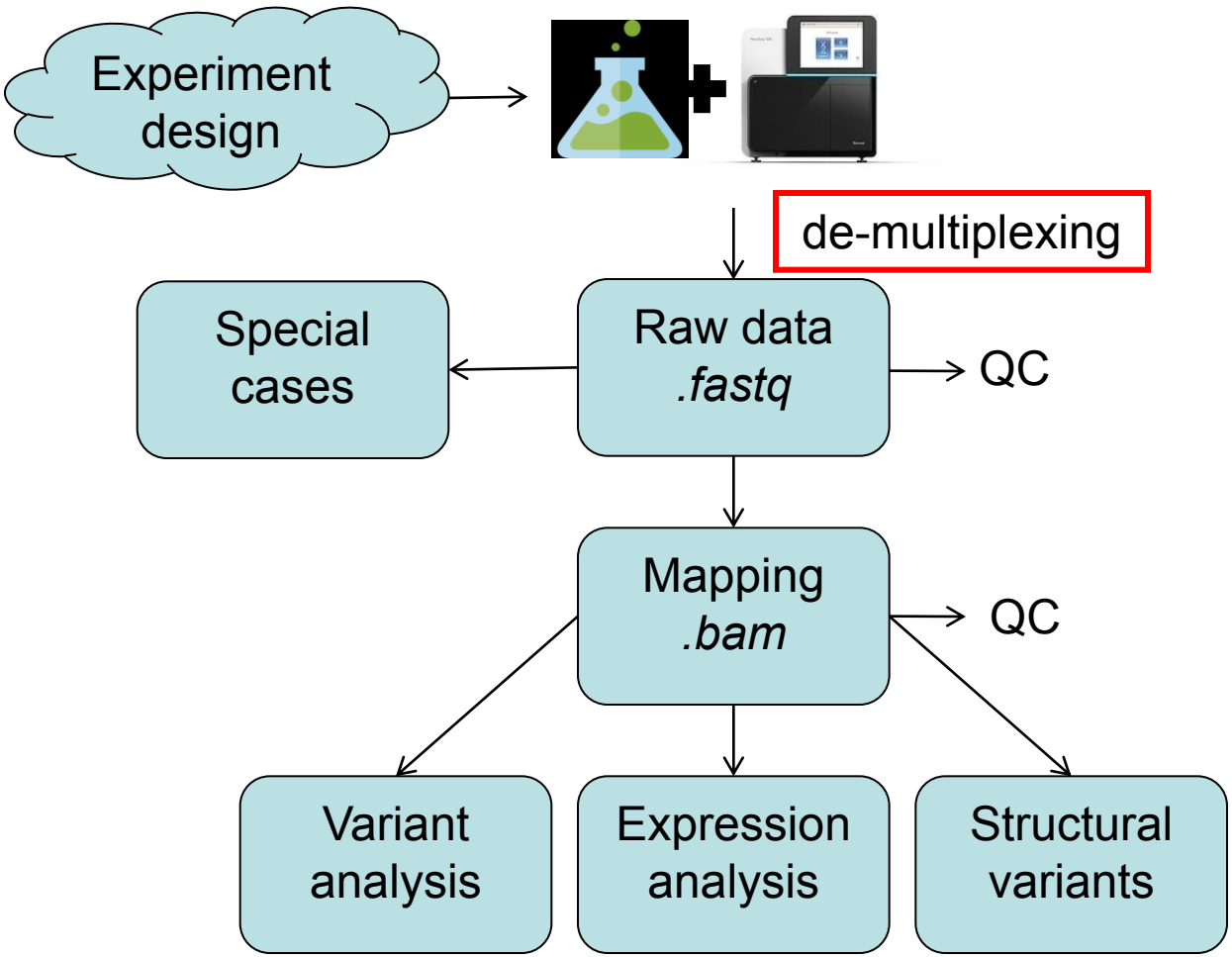


Experimental design

- **Have a hypotheses!**
- **Consult with sequencing expert and bioinformatician**

1. **If experiment you have in mind can be done in a way you are planning to.**
2. **If the results you want can be obtained from the planned sequencing. (desired outcome)**
3. **If the bioinformatician knows how to perform specific types of analyses and how long it will probably take.**

NGS data analysis

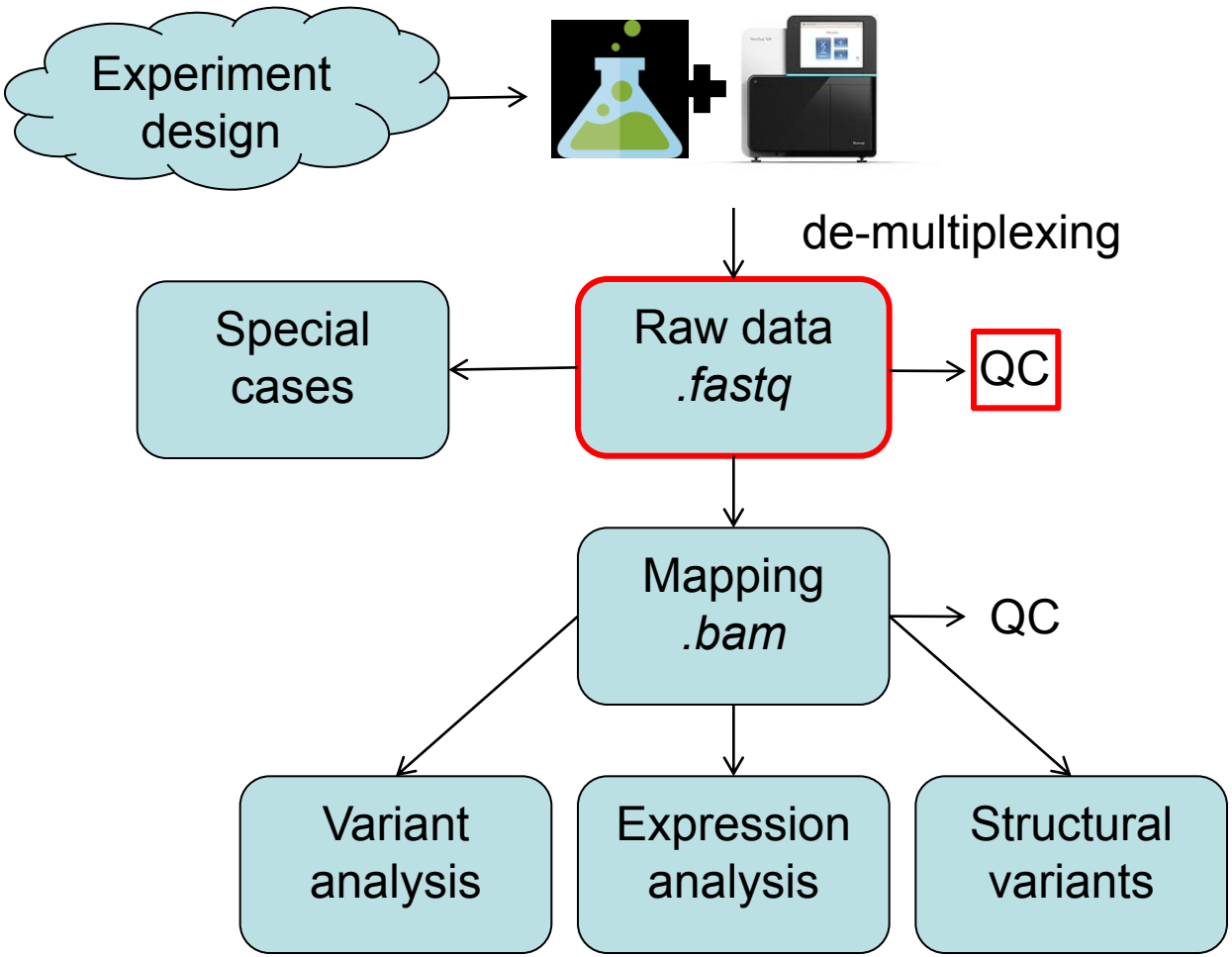


De-multiplexing

- **Not perfect**
  - **In silico contamination – problem for MRD detection**

- **Sample naming and organisation**
- **Naming**
  - **Unique names**
  - **_ vs – vs .**
  - **Special characters: $&|@+- …**
  - **Really tricky: - vs −**
- **Organization**
  - **Should not be your worries**
  - **For any longer ‘operation’ comprehensive database is necessary**
  - **Currently working on it ourselves ☺**
  - **Please fill the forms carefully and as much as possible**

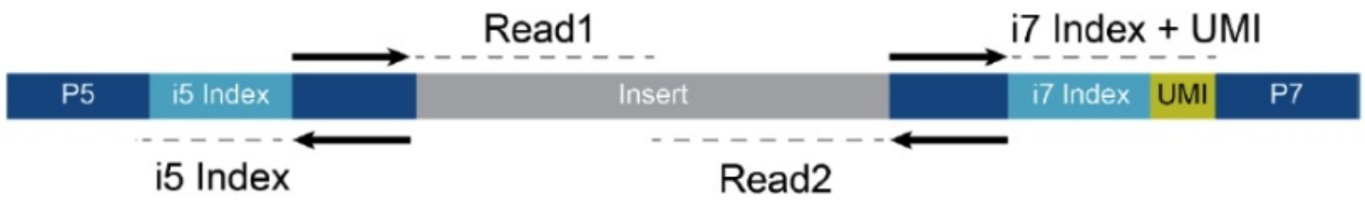
NGS data analysis



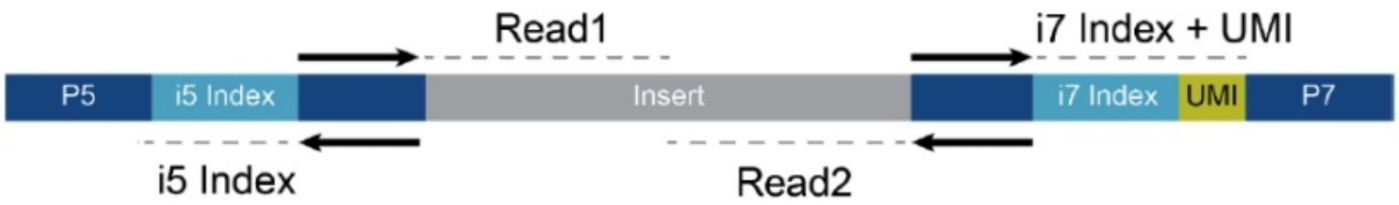
Data pre-processing

- Primer (adaptor) trimming
  - To cut adapter usually not necessary but good practice
  - Primer removal is necessary

- UMI extraction



UMI – unique molecular identifiers



- Each molecular fragment gets unique n-base sequence (n ~ 8-12)
- Usage:
  - Mark duplicates
  - Consensus sequence
    - sequencing (PCR) error removal

Raw data - QC

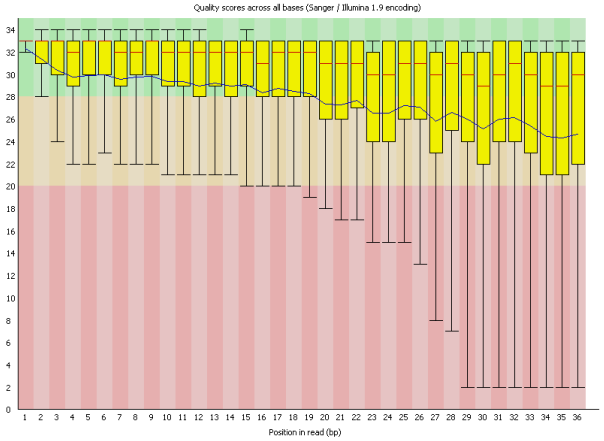
- **Fastq - q stands for quality – coded phred score**

```
CFFFFEFFGCEEGECFGGGGAFF87@E:++6C<++3:,8,33,,:,,,:,,:,,,
```

$$Q = -10 \cdot \log_{10} P$$

Quality	Error probability
5	31%
10	10%
20	1%
30	0.1%

- **Very good for early problem detection**
- **Reasonable for trimming and read filtering**
  - **RNA seq - above phred score 5**
- **Not good for individual variant analysis**



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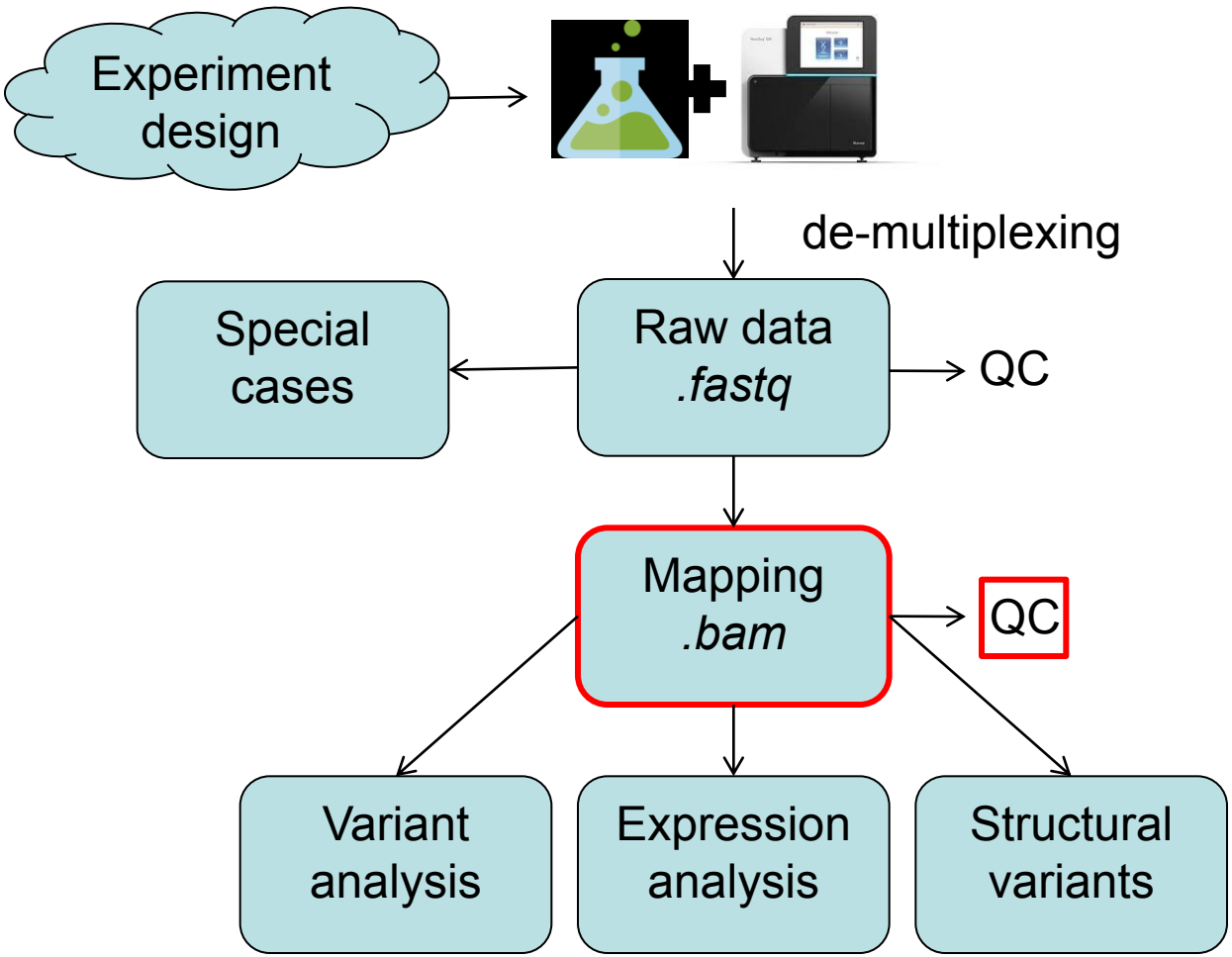
NGS data analysis



Alignment

- **Computationally most demanding**
- **More or less standardized**

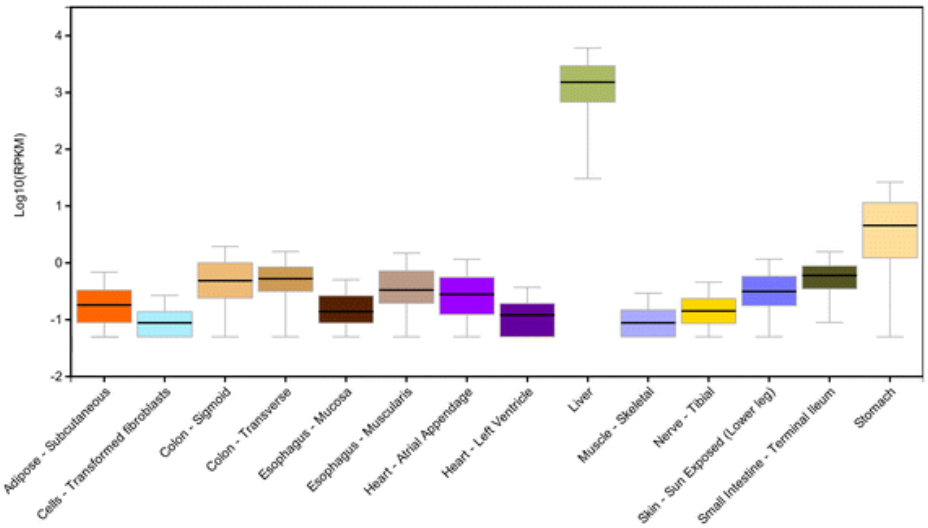
- **Align to genome then select region of interest (ROI) <- .bed file**
  - **Don’t force alignment**
  - **Keep the information about wrongly aligned for QC**
  - **Exception targeted SV detection**

- **Our standard procedure:**
  - **BWA - DNA**
  - **STAR - RNA**
  - **Chimira – sRNA**

Alignment - QC

- **DNA**
  - **Mean coverage and variance**
  - **Percentage of covered with at least**
    - **In WES we define good quality if at lest 90% of positions are covered at least 20x**
  - **Per base coverage – in smaller experiments**

- **RNA**
  - **Per gene coverage**
  - **Variability of per gene mapping**
  - **Gene counts distribution**
  - **rRNA content estimate**
  - **Tissue expression check - gtex**



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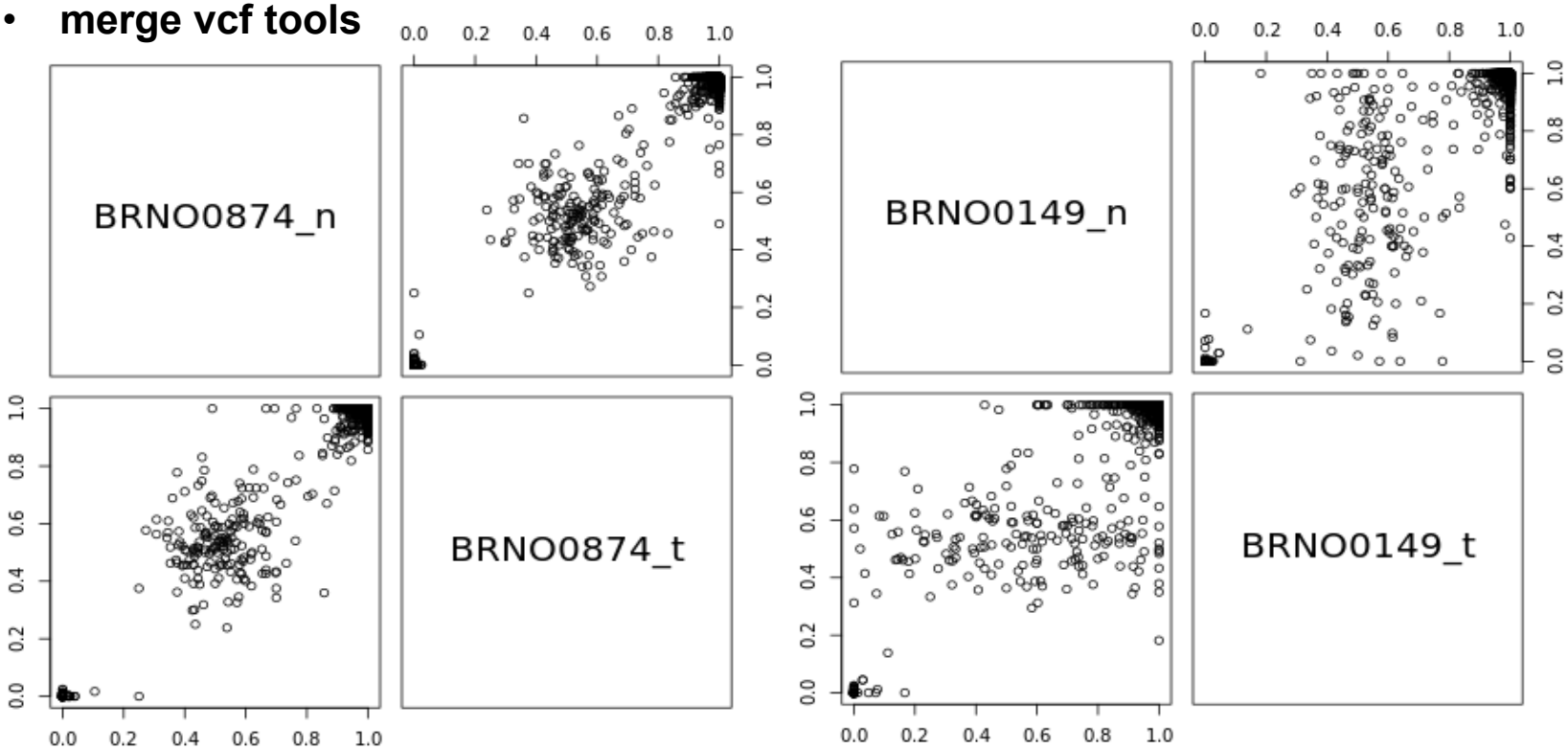
Alignment - QC

- **BAM cross-contamination**
  - **verifyBamID**
  - **FREEMIX - bellow 0.03 = OK**
- **Cross-sample snp allele frequency correlation**
  - **merge vcf tools**



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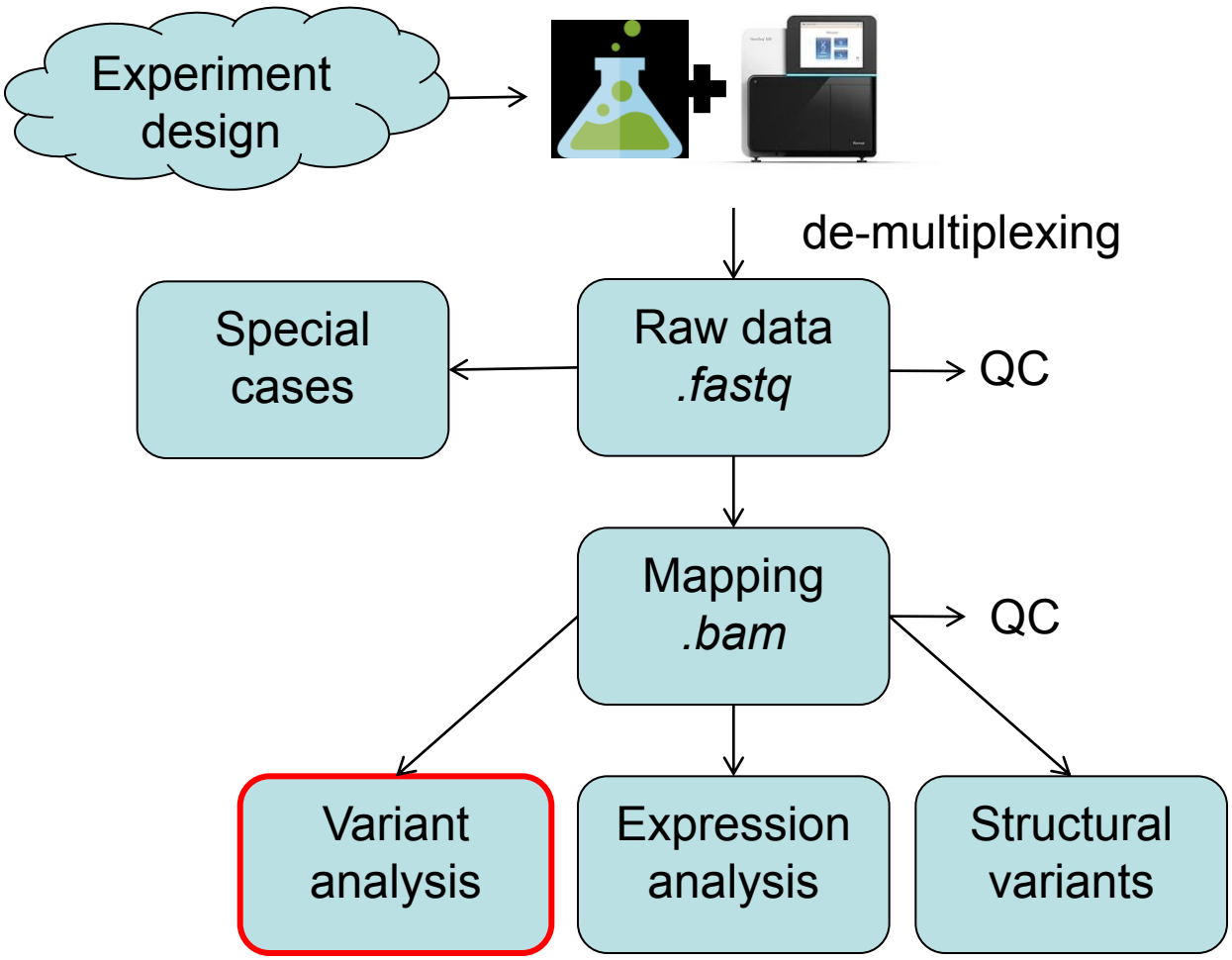
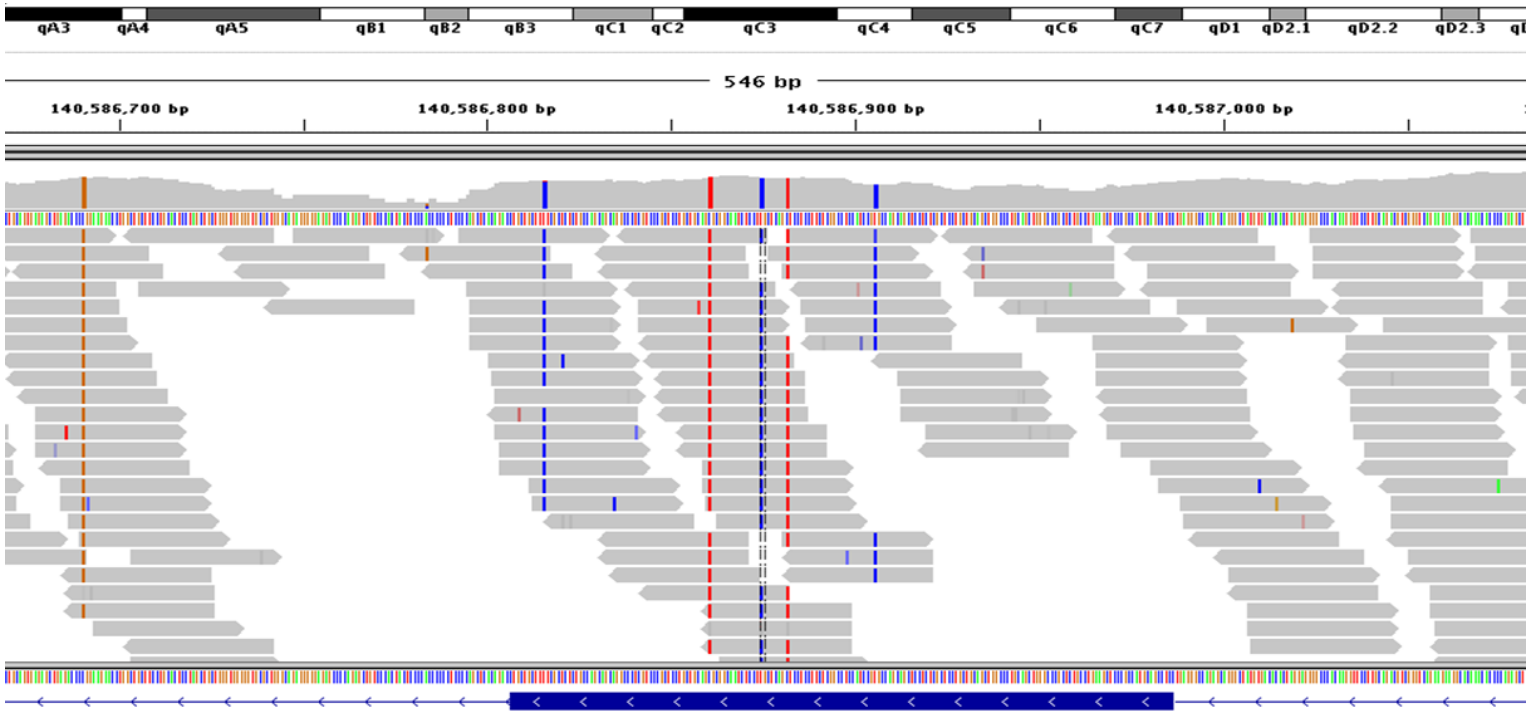
NGS data analysis



Variant Calling



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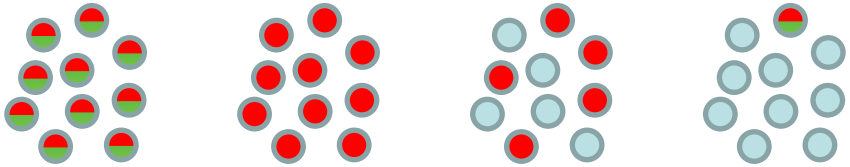
Variant Calling

- Type of comparison
  - ermline – to reference genome
  - Somatic – to other sample(s)
- Expected variant heterogeneity
  - Indirectly corelates to the necessary coverage



Variant Calling - planning

- **Scope**

Scope	genes	~bp	~% of WG
WGS	~22000	3 200 mil	100%
WES	22000	30 mil	1%
PanCancer	1049	1.2 mil	0.04%
CZECANCA	219	250 000	0.0083%
TP53	1	25772	0.000859%

- **With fixed cost of bp-read it seems the price is linear**
- **The "price" of the analysis must be considered**
  - **Power of the results (sensitivity, specificity)**

Variant Calling - planning

- **Example**

  Lets have an analysis with per base false positive error rate 0.0001.

  Resulsts in:

  2 false variants in TP53 gene

  3000 false variants in WES!

- **WES on a single healthy person with a question:**

  Are there any variants?

- **Answer is YES**

Variant Calling - planning

- **Sample design**
  - **Germline**
  - **Somatic**
  - **Tumor - Normal**
  - **Family**

- **Any relationship between samples for comparison improve specificity dramatically**
  - **Not sensitivity**
- **Somatic variant calling without normal needs high coverage**

- **RNA**
  - **Depends on gene expression levels**
  - **Variant might not be there! – gtex, previous runs QC**

Variant Calling

- **Specificity vs. Sensitivity**
- **Tools**
  - **varscan – no statististics = no assumptions**
  - **vardict**
  - **gatk haplotype caller**
  - **mutect – only snp**
  - **pindel – only indels**
  - **freebayes**
- **Callers combining – usual strategy**
- **Variant Annotation**
  - **Annovar – good database**
  - **snpEff**
  - **vep – variant effect predictor**

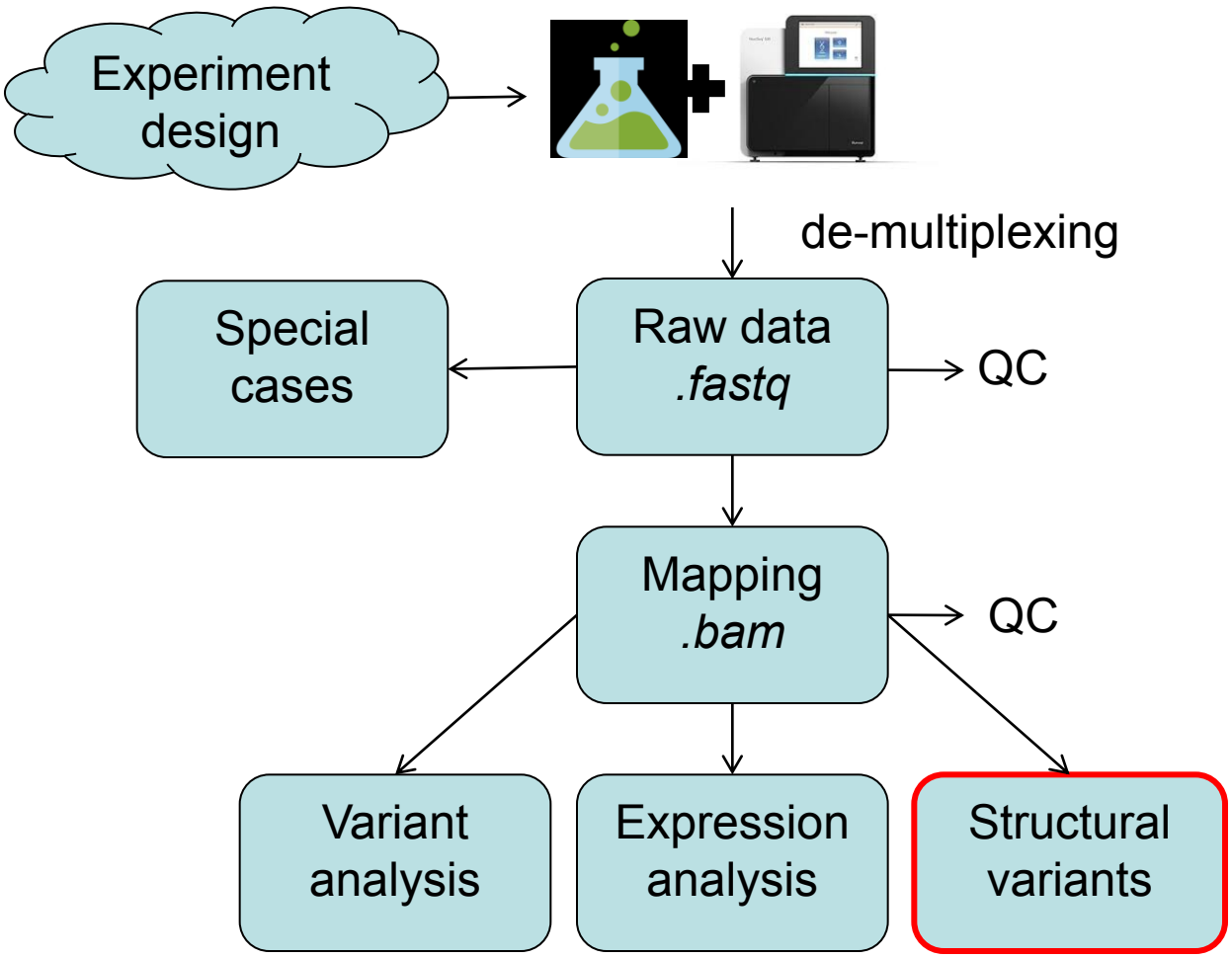
Variant Calling

- **Variant annotation can help variant calling significantly**
- **Variant occurrence in normal population**
  - **1000 genome project – above 5%**
- **Variant consequences cut off**

*	SO term	SO description	SO accession	Display term	IMPACT
	transcript_ablation	A feature ablation whereby the deleted region includes a transcript feature	SO:0001893	Transcript ablation	HIGH
	splice_acceptor_variant	A splice variant that changes the 2 base region at the 3' end of an intron	SO:0001574	Splice acceptor variant	HIGH
	splice_donor_variant	A splice variant that changes the 2 base region at the 5' end of an intron	SO:0001575	Splice donor variant	HIGH
	stop_gained	A sequence variant whereby at least one base of a codon is changed, resulting in a premature stop codon, leading to a shortened transcript	SO:0001587	Stop gained	HIGH
	frameshift_variant	A sequence variant which causes a disruption of the translational reading frame, because the number of nucleotides inserted or deleted is not a multiple of three	SO:0001589	Frameshift variant	HIGH
	stop_lost	A sequence variant where at least one base of the terminator codon (stop) is changed, resulting in an elongated transcript	SO:0001578	Stop lost	HIGH
	start_lost	A codon variant that changes at least one base of the canonical start codo	SO:0002012	Start lost	HIGH
	transcript_amplification	A feature amplification of a region containing a transcript	SO:0001889	Transcript amplification	HIGH
	inframe_insertion	An inframe non synonymous variant that inserts bases into in the coding sequenc	SO:0001821	Inframe insertion	MODERATE
	inframe_deletion	An inframe non synonymous variant that deletes bases from the coding sequenc	SO:0001822	Inframe deletion	MODERATE
	missense_variant	A sequence variant, that changes one or more bases, resulting in a different amino acid sequence but where the length is preserved	SO:0001583	Missense variant	MODERATE
	protein_altering_variant	A sequence_variant which is predicted to change the protein encoded in the coding sequence	SO:0001818	Protein altering variant	MODERATE
	splice_region_variant	A sequence variant in which a change has occurred within the region of the splice site, either within 1-3 bases of the exon or 3-8 bases of the intron	SO:0001630	Splice region variant	LOW
	incomplete_terminal_codon_variant	A sequence variant where at least one base of the final codon of an incompletely annotated transcript is changed	SO:0001626	Incomplete terminal codon variant	LOW
	stop_retained_variant	A sequence variant where at least one base in the terminator codon is changed, but the terminator remains	SO:0001567	Stop retained variant	LOW
	synonymous_variant	A sequence variant where there is no resulting change to the encoded amino acid	SO:0001819	Synonymous variant	LOW

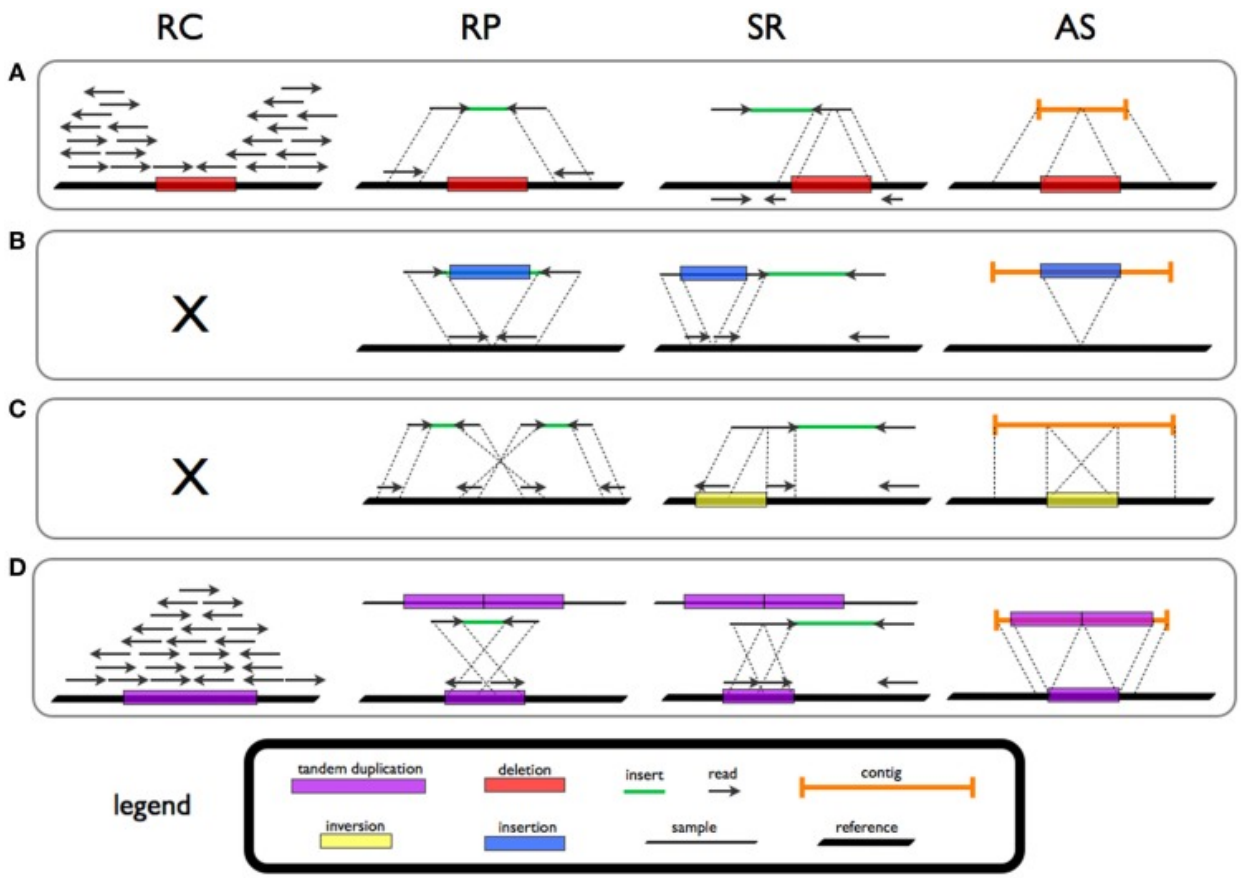
- **Database can help significantly – Sophia Genetics**

NGS data analysis



Structural variants

- **discordant read(-pairs) mapping**
- **copy number variants (CNV)**



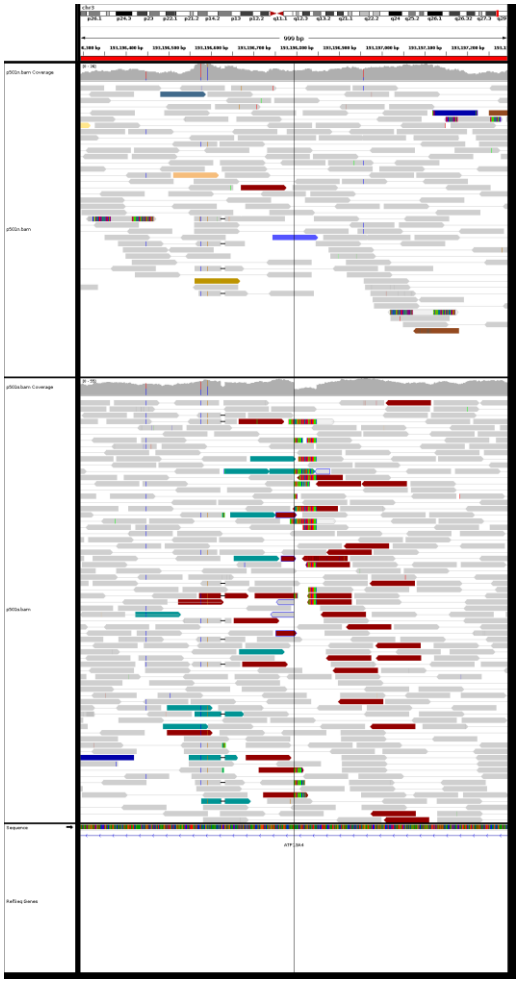
Structural variants

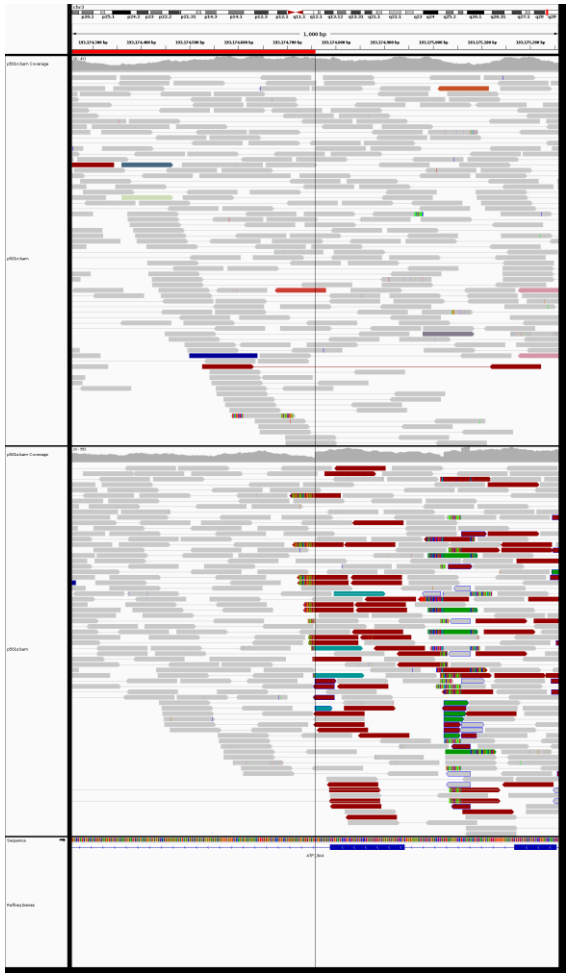
- **CNV**
- **long variants in WGS – ControlFreec**
- **Smaller variants for WES / target panel**
  - **Somatic – tumor,normal**
  - **Germline - lot of references**
    - **XHMM**

- **Read-pairs very noisy expect a lot of FP**
- **BreakPoint**
  - **Target panel with short reads**
- **Delly**
  - **everything else**

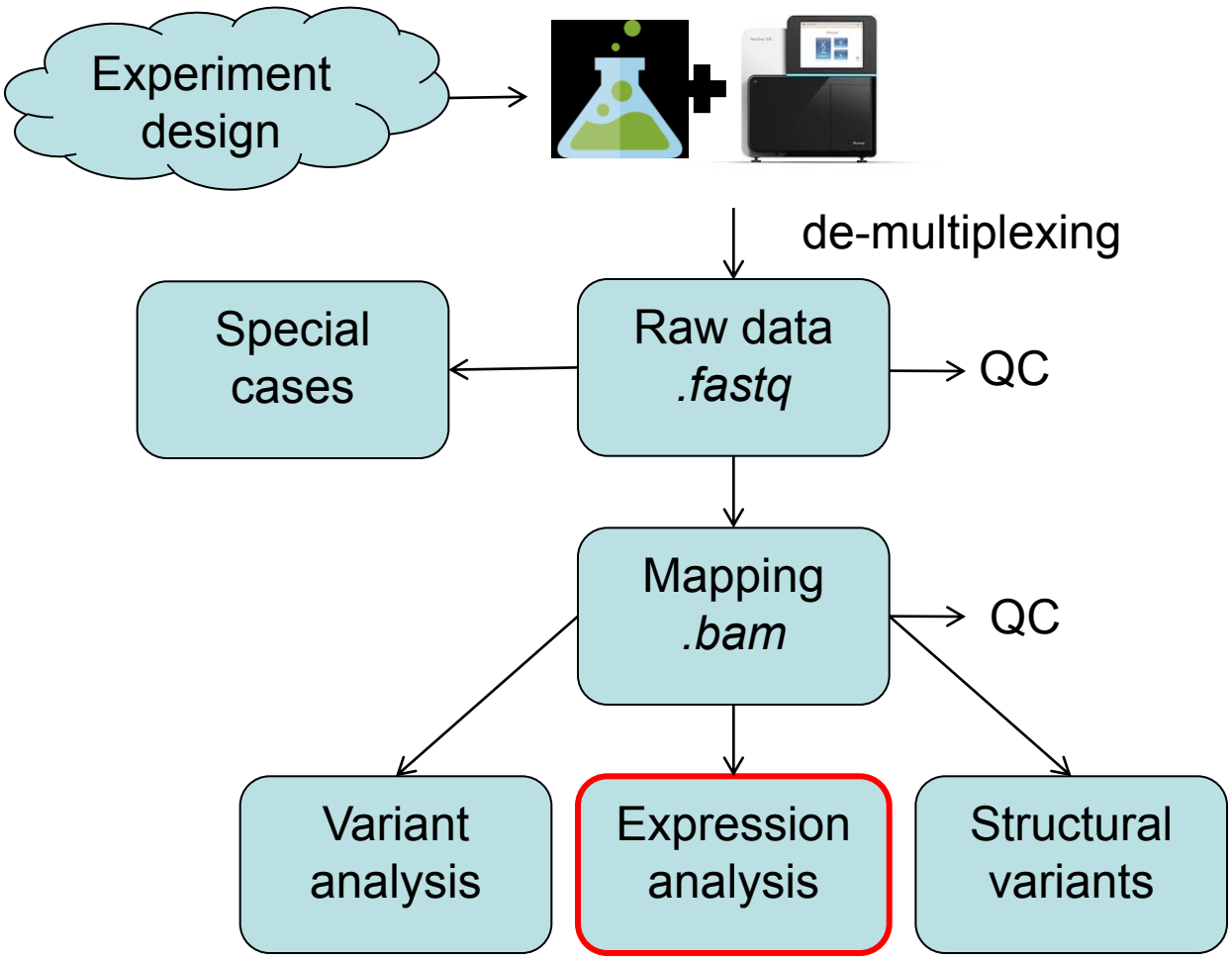
Structural variants

- **Manual check with IGV (batchmode)**

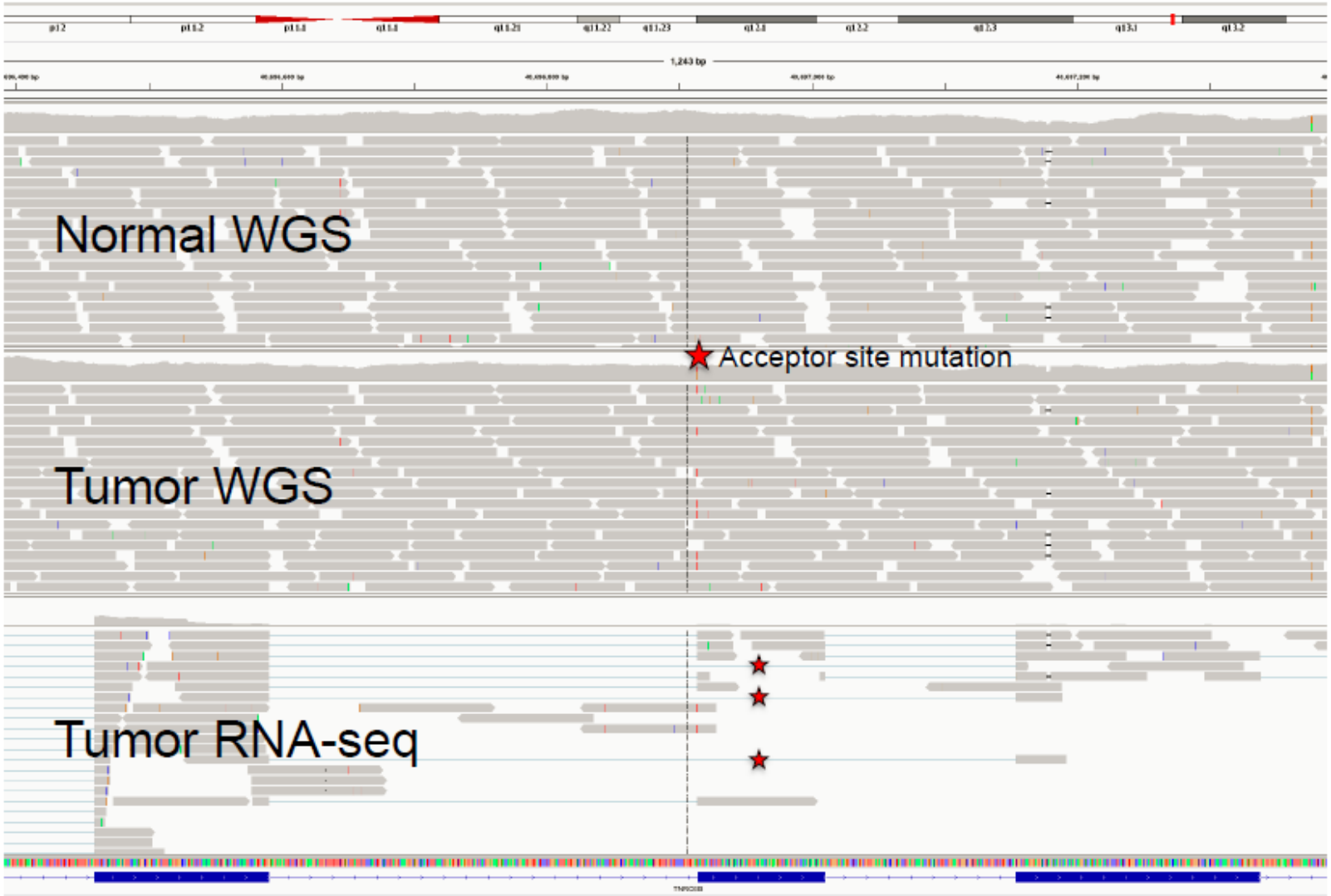




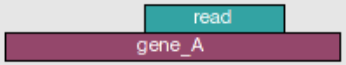
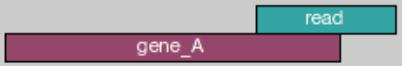
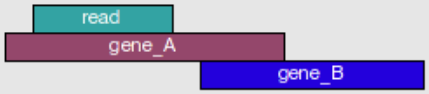
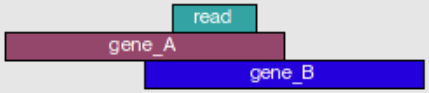
NGS data analysis



Expression analysis



Counting schemes

	union	intersection _strict	intersection _nonempty
	gene_A	gene_A	gene_A
	gene_A	no_feature	gene_A
	gene_A	no_feature	gene_A
	gene_A	gene_A	gene_A
	gene_A	gene_A	gene_A
	ambiguous	gene_A	gene_A
	ambiguous	ambiguous	ambiguous

Expression analysis - planning

- **3 way balance**
  - **Read depth**
  - **Biological replicates**
  - **Fold change (number of genes) sensitivity**

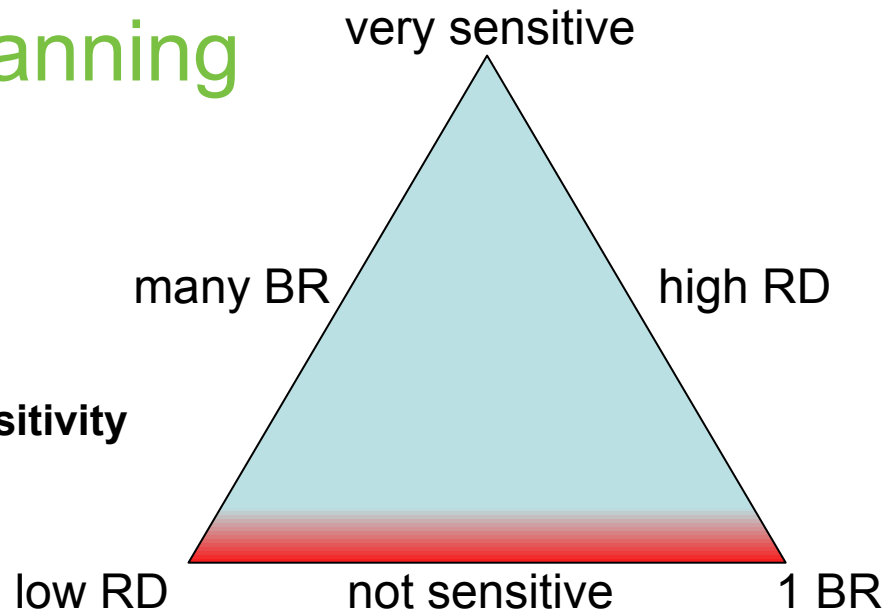
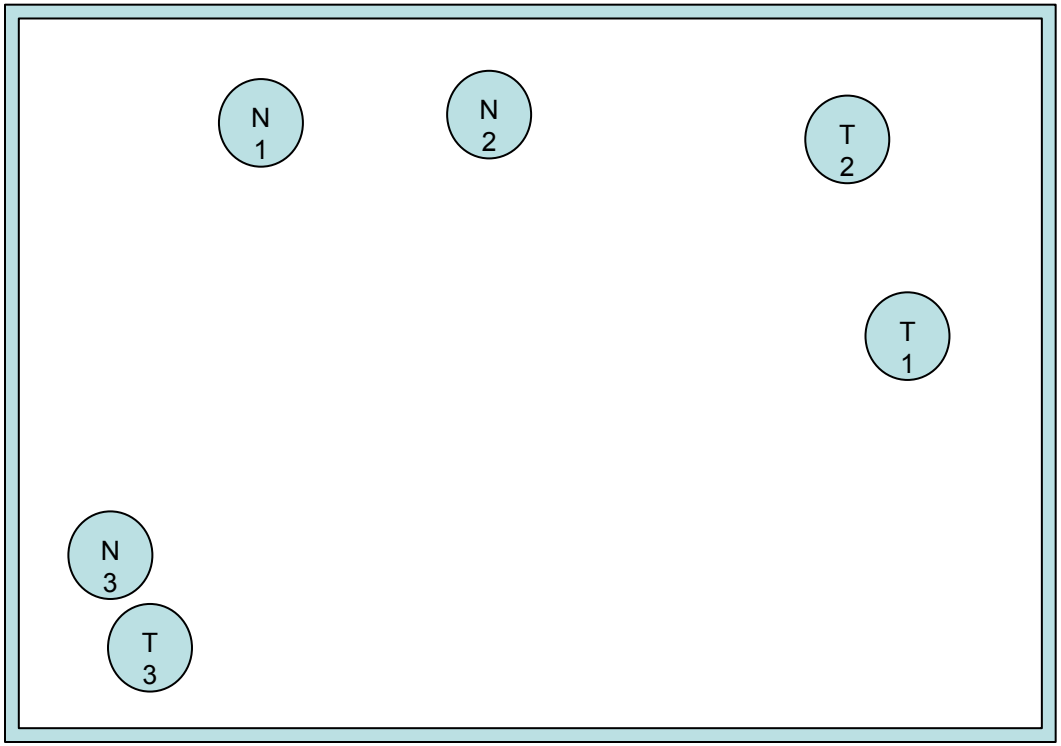


Table 1
Statistical power to detect differential expression varies with effect size, sequencing depth and number of replicates

	Replicates per group		
	3	5	10
Effect size (fold change)			
1.25	17 %	25 %	44 %
1.5	43 %	64 %	91 %
2	87 %	98 %	100 %
Sequencing depth (millions of reads)			
3	19 %	29 %	52 %
10	33 %	51 %	80 %
15	38 %	57 %	85 %

Expression analysis - planning

- **Replicates**
- **Technical vs. biological**
  - **Technical only for technique testing**
- **Highly suggested minimum = 4 rep**



Expression analysis - planning

- **Depth**
- **Human ~ 22 000 genes = minimum 20 mil mapped reads**
- **Good 25 mil mapped reads**

- **Mapped reads!**
  - **rRNA removal**
  - **Size selection for sRNA**

- **Trade-off**

4 replicates with 20 mil vs. 3 replicates with 30 mil

9 replicates with 25 mil vs. 10 replicates with 20 mil

Expression analysis - planning

- **Depth**
- **Human ~ 22 000 genes = minimum 20 mil mapped reads**
- **Good 25 mil mapped reads**

- **Mapped reads!**
  - **rRNA removal – 90% rRNA**
  - **Size selection for sRNA**

- **Trade-off**

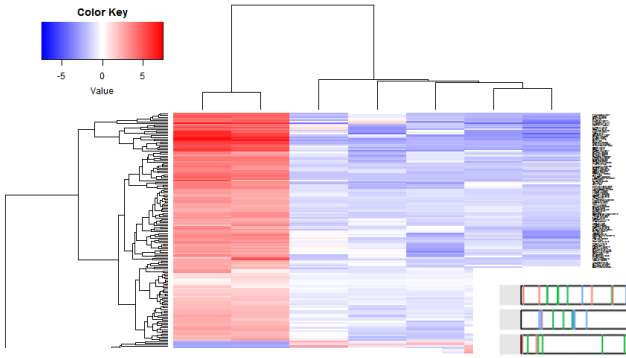
4 replicates with 20 mil vs. 3 replicates with 30 mil

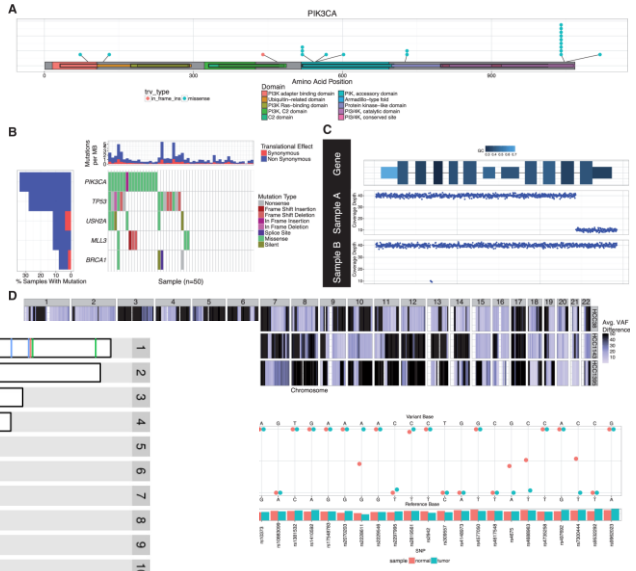
9 replicates with 25 mil vs. 10 replicates with 20 mil

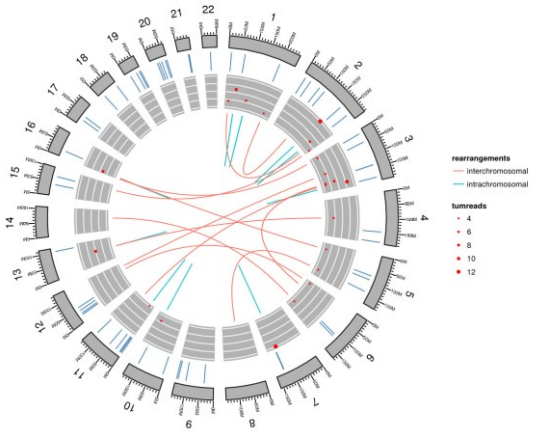
Reality: 3 replicates with 15 mil reads

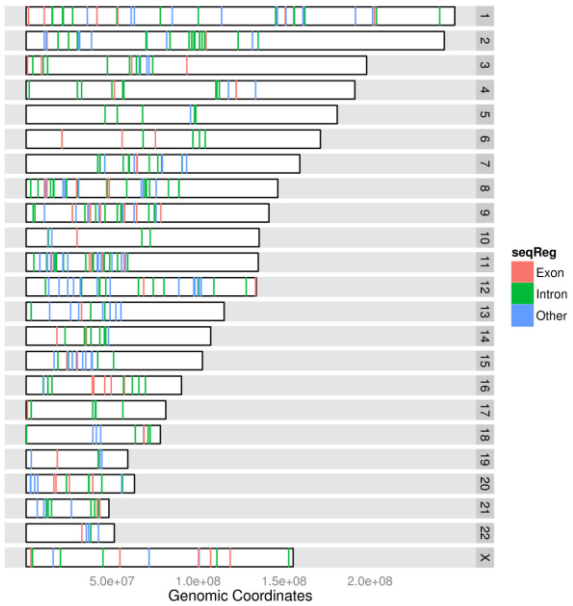
Visualization

- **Bioinformatician provide all he think might be helpful**
- **Researcher requests exactly what he wants**
- **Good to find a balance**









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Thank you for your attention

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