



# DNA/RNA EXTRACTION & LIBRARY CONSTRUCTION FOR METAGENOMIC STUDY

Science discussion Aug 8<sup>th</sup> 2024

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# Sample To Insight



## 1 Part 1: OVERVIEW

- metagenomic
- Research workflow

## 2 Part 2: SAMPLE MANAGEMENT

- Element Of Samples Management
- Biomolecule Stability
- Transport & Storage Of Sample Types

## 3 Part 3: DISRUPTION

## 4 Part 4: PURIFICATION

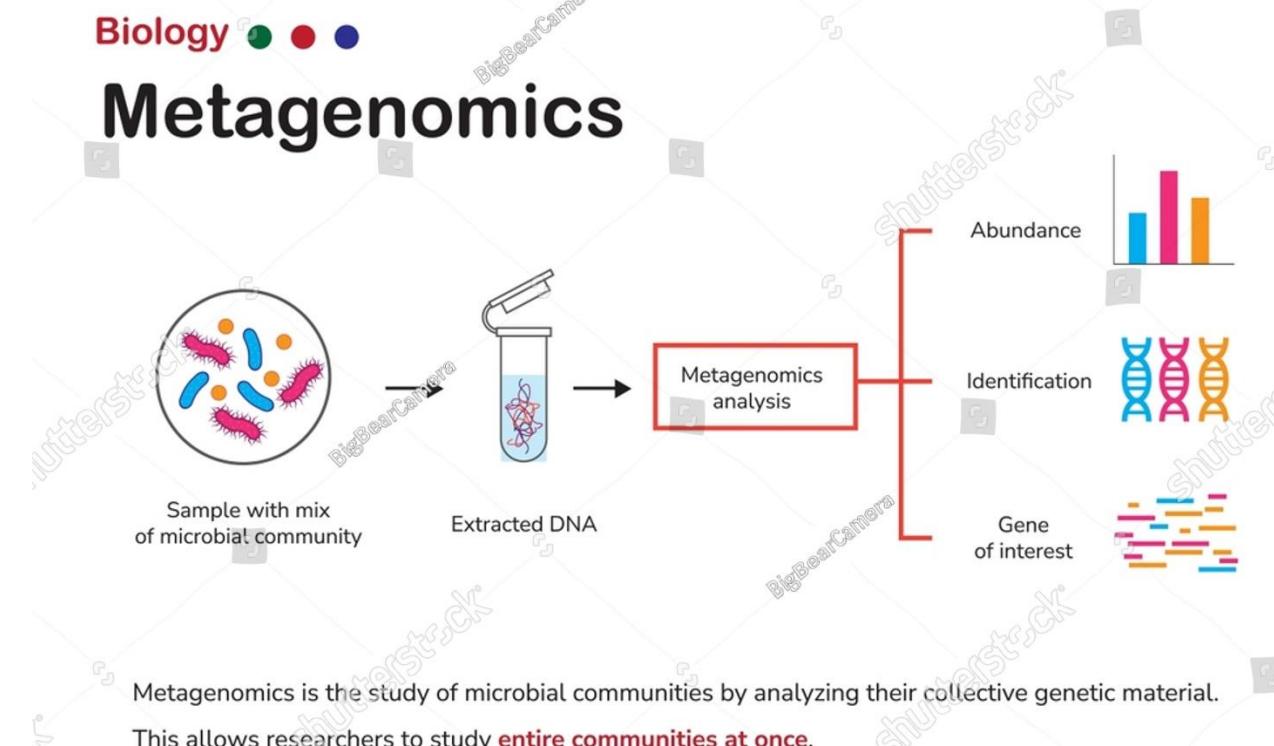
- Challanges
- Homebrews vs Modern Technologies
- Optimizing Extraction for Accurate Results

## 5 Part 5: QIAGEN SOLUTION

## 6 Part 6: NGS library construction

# METAGENOMIC

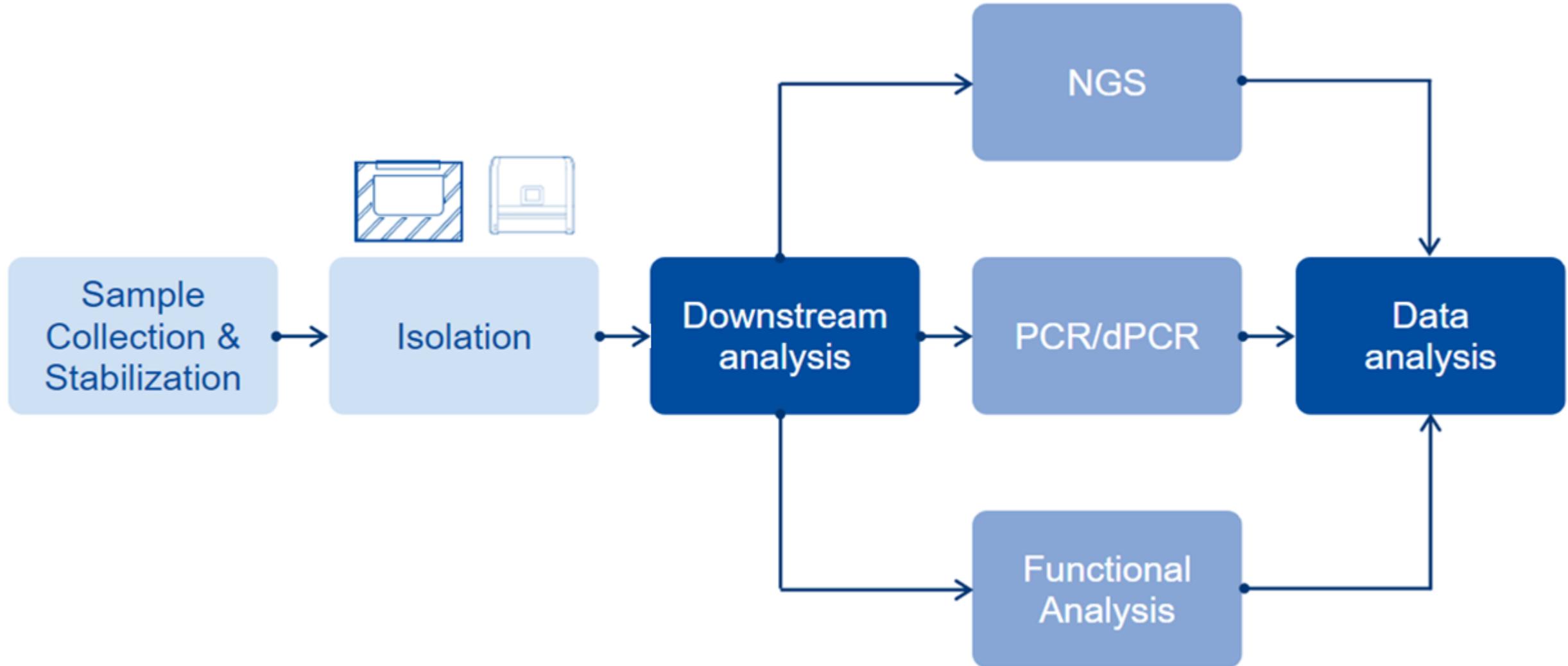
- the DNA from a community of microorganisms in a given environment
- No focusing in single target
- analyze the genomes of multiple organisms, particularly microbes, simultaneously



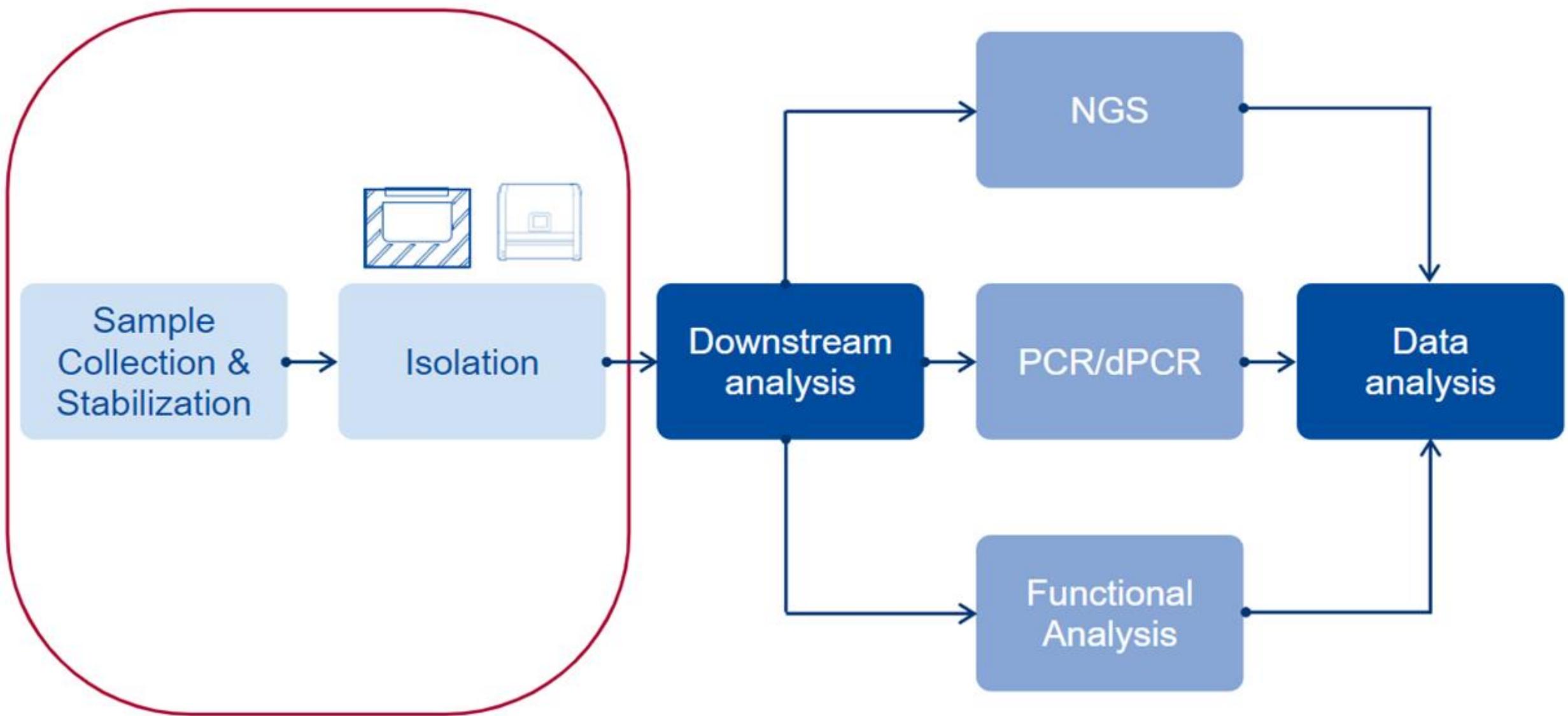
shutterstock

IMAGE ID: 2266470377  
[www.shutterstock.com](http://www.shutterstock.com)

# RESEARCH WORKFLOW



it all starts with the sample!



# RESEARCH WORKFLOW



A typical customer workflow begins with the **management of the sample** and is followed by **disruption and purification**.



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QIAGEN offers **solutions** at every step of this workflow. For this self-study unit, we will be focused on the two parts of **sample technologies**:

is

- Sample management
- Disruption and purification of the sample

# Let's start with the introduction to sample management technologies



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→ Homebrews vs Modern Technologies

→ Optimizing Extraction for Accurate Results

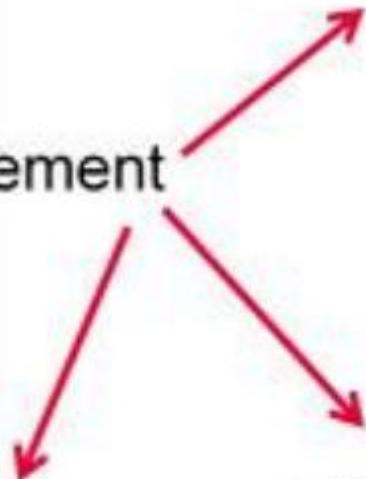
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# ELEMENT OF SAMPLES MANAGEMENT



Sample management involves...



Choosing a sample source and collection method

Transport and storage



Stabilization



# CHALLENGES



The overall challenges a researcher faces when dealing with samples are:

**Stability of biomolecule desired  
(degradation)**

**Space and equipment**

**Standardization**

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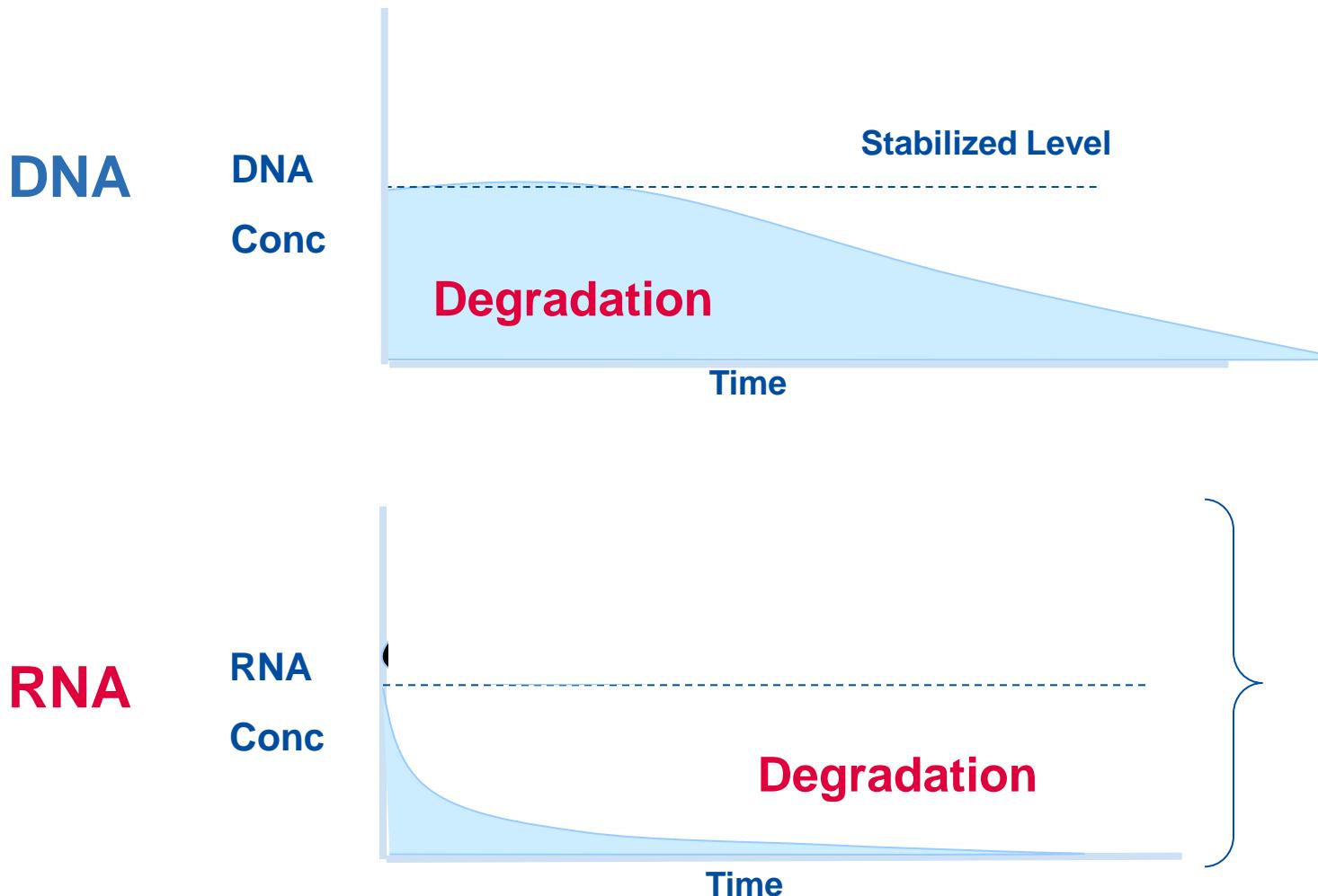
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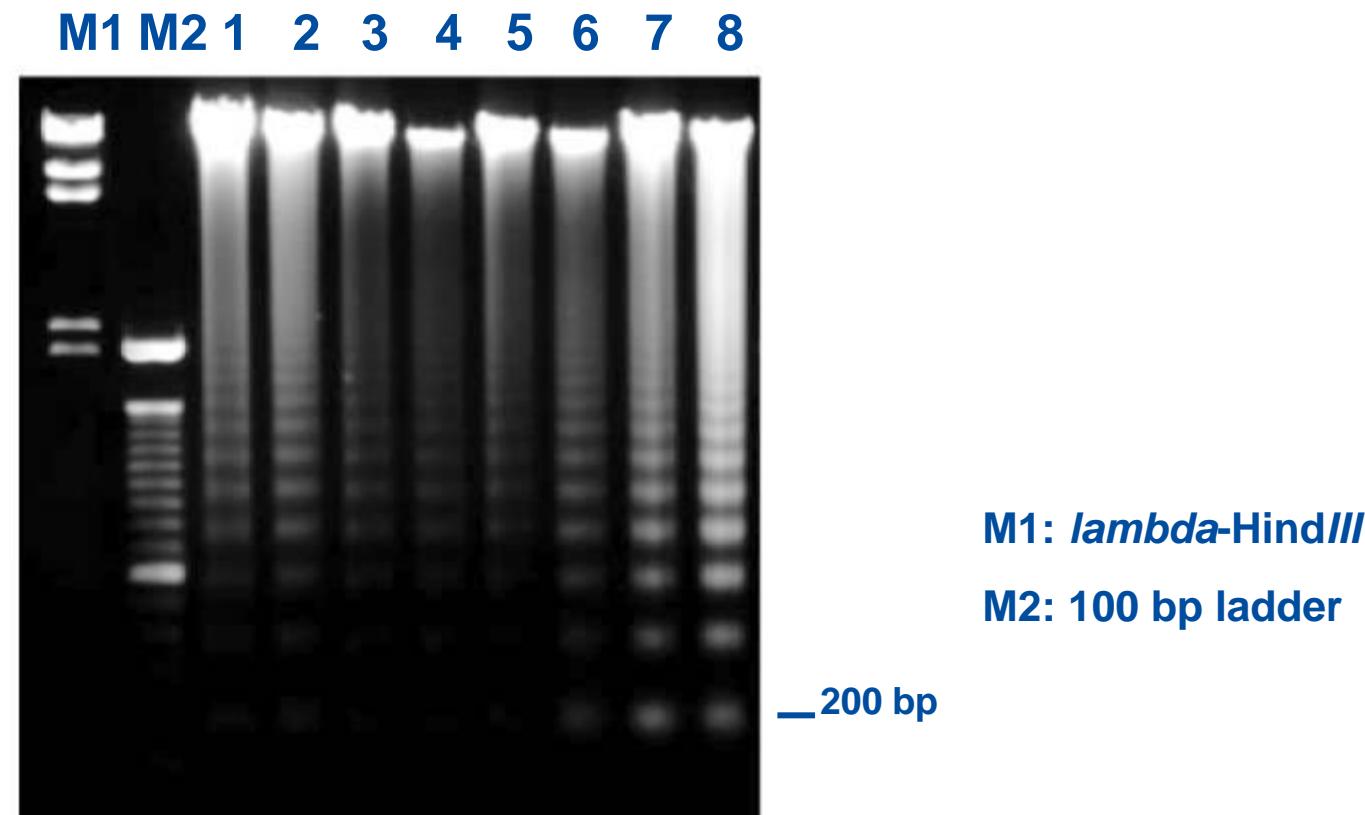
# DNA vs RNA STABILITY



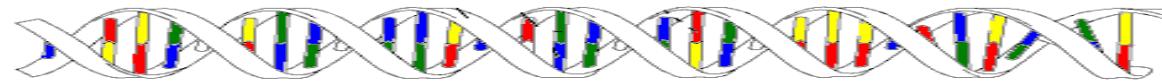
# DNA STABILITY



**Apoptotic Banding in stored blood:  
8 blood samples, 1 week, 4°C**



# INTEGRITY DNA IN THE SAMPLE



## DNA Length

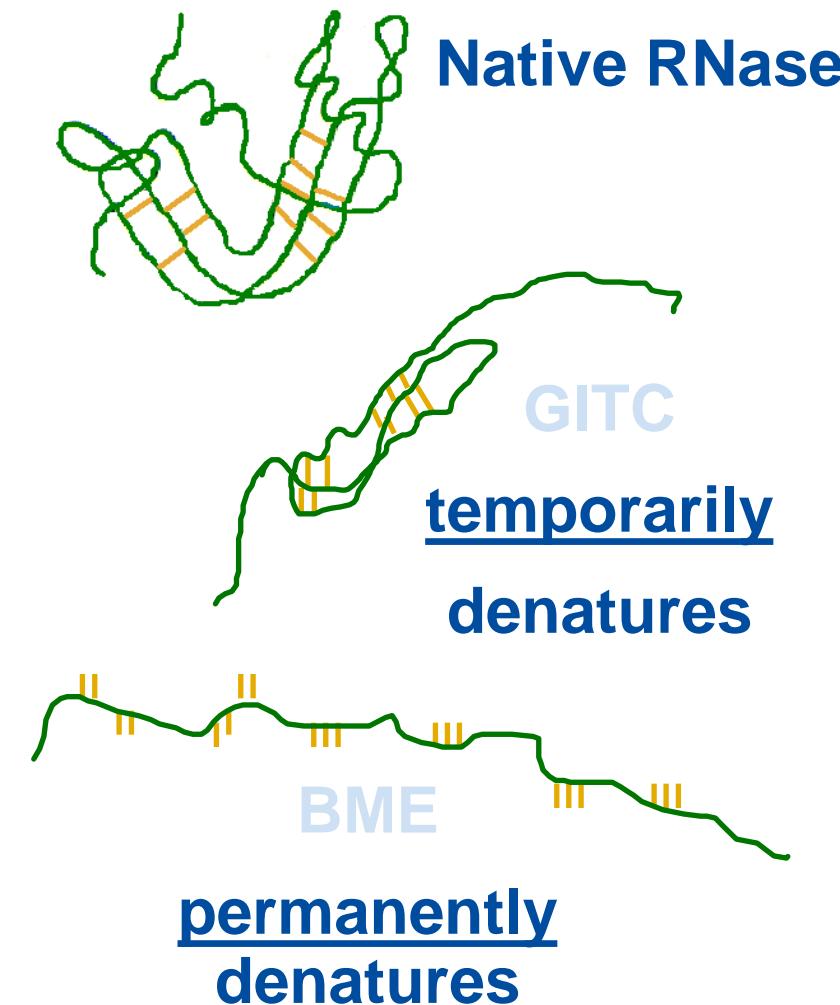
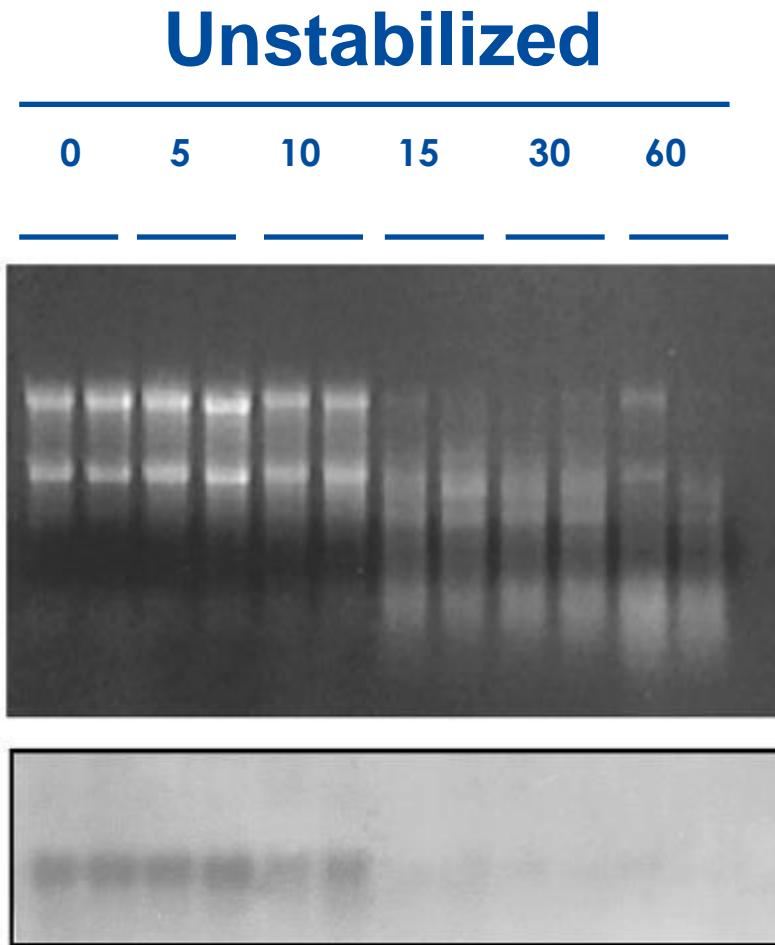
**Critical**

- PCR/qPCR/dPCR
- NGS long reads
- RFLP

**Non-critical**

- NGS short reads

# RNA STABILITY



- Chaotropic salt
- Reducing agents
- Inhibitors
- Extreme cold

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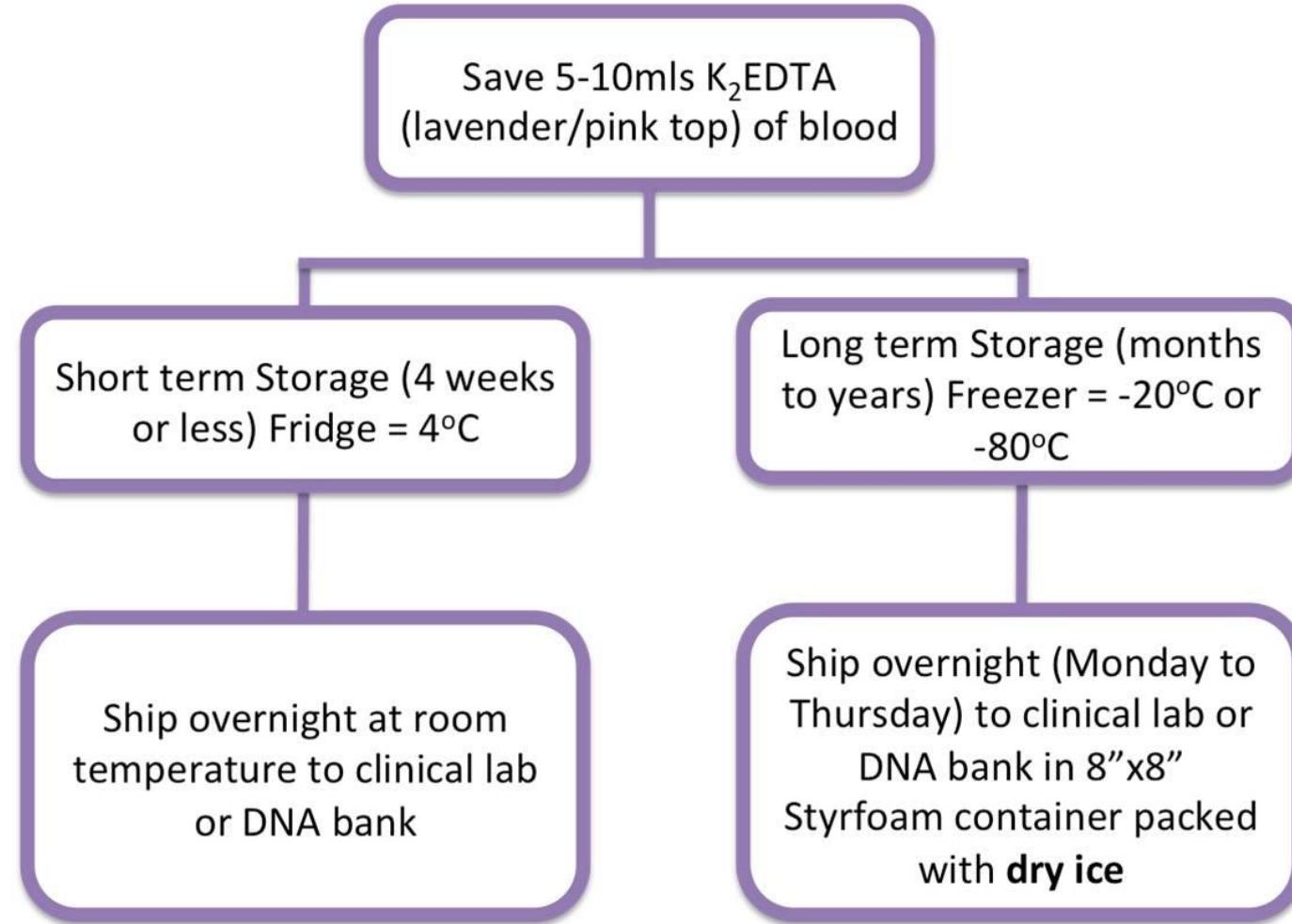
## 6 Part 6: NGS library construction

# BLOOD SAMPLE

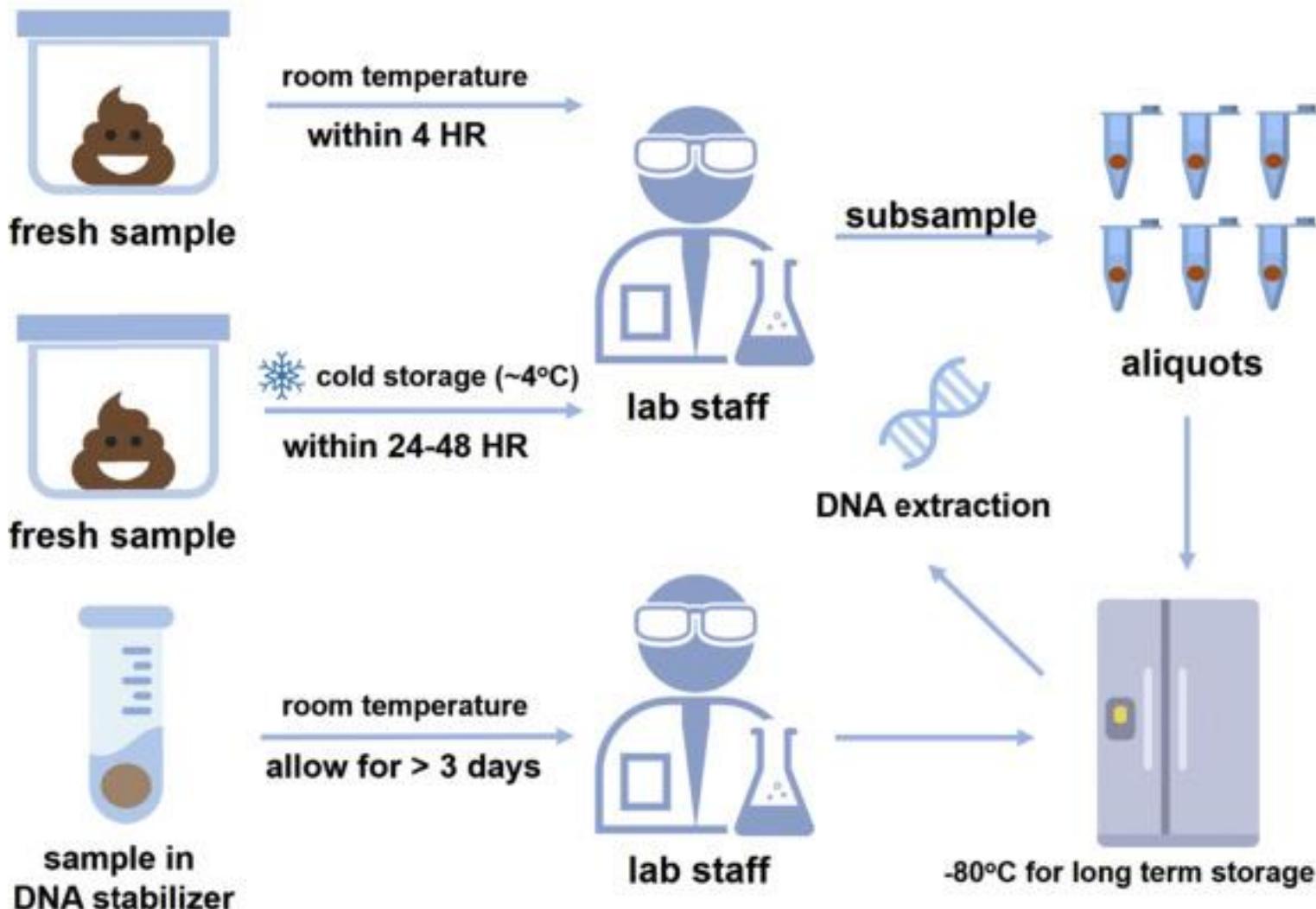


## After blood collection

## Sample Transport



# STOOL SAMPLE



# SOIL SAMPLE



- Soil is complex and diverse, making it one of the most challenging sample types for microbial nucleic acid isolation
- soil produces humic substances which is strong inhibitor
- Humic substances are a complex and heterogenous mix of organic molecules
- Can co-purify with DNA/RNA
- Another things: heavy metals or polysaccharides or Nuclease content



Sieve soil samples to normalize texture and remove large particles. This increases DNA yield consistency.



After collection, use your sample as soon as possible. If storage is required we recommend storing the sample at -70 to -20°C, avoiding thaw/freeze cycles.

# OTHER SAMPLE TYPE

Sample Type	Sample Management
FFPE	<ul style="list-style-type: none"> <li>Importance of quick tissue removal and fixation</li> <li>Use of 10% formalin solution (3.7% formaldehyde, 1–1.5% methanol)</li> <li>Optimal use of neutral-buffered formalin</li> <li>Ratio of formalin to tissue: at least 10:1</li> <li>Avoid overfixation: no more than 24 hours</li> <li>Use fresh alcohol and xylene for optimal results</li> <li>Avoid additives in paraffin</li> <li><b>Storage:</b> RT for years</li> <li><b>Transport:</b> RT</li> </ul>
Animal Tissue	<ul style="list-style-type: none"> <li><b>Fresh:</b> Storage: –20°C, –80°C, or in liquid nitrogen</li> <li><b>Lysed Tissue :</b> ambient temperature</li> </ul>
Animal, Yeast, and Bacterial Cell Cultures	<ul style="list-style-type: none"> <li><b>Centrifuge, remove supernatant, store at –20°C or –80°C</b></li> </ul>
Plant Tissue	<ul style="list-style-type: none"> <li><b>Fresh leaves/needles:</b> Up to 24 hours at 4°C</li> <li><b>Longer-term storage:</b> –80°C</li> <li><b>Drying methods:</b> Silica gel, food dehydrators, lyophilizers</li> </ul>

# STABILIZATION

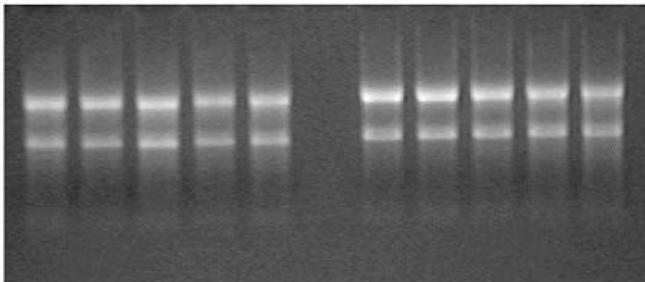


# Liquid

## Lung

C 3 months, -20°C  
4 weeks, 4°C  
7 days, RT  
24 hours, 37°C

C 3 months, -20°C  
4 weeks, 4°C  
7 days, RT  
24 hours, 37°C



# Storage

3 day at 37°C

7 days at 18-25°C

**4 weeks at 2-8°C**

- GAPDH

**Archive: -20°C - -80°C**

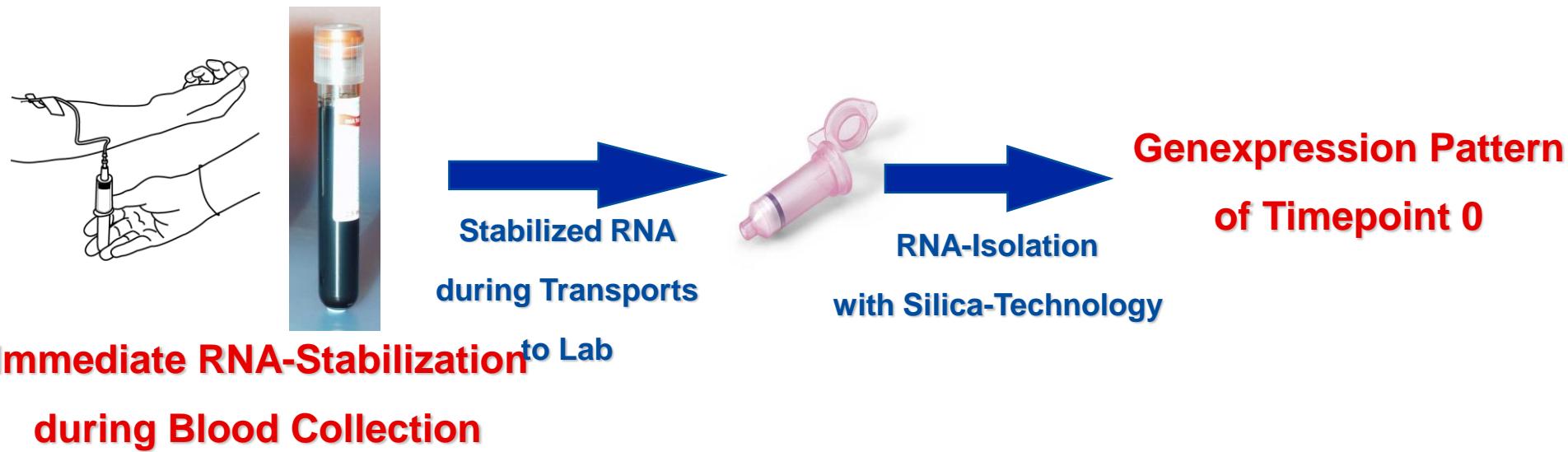
# Tube system



- RNA stabilization for up to 3 days at 18–25°C
  - DNA stabilization for up to 14 days at 18–25°C
  - Stabilization for at least 60 months (RNA) & Years (DNA) at –20°C or –70°C

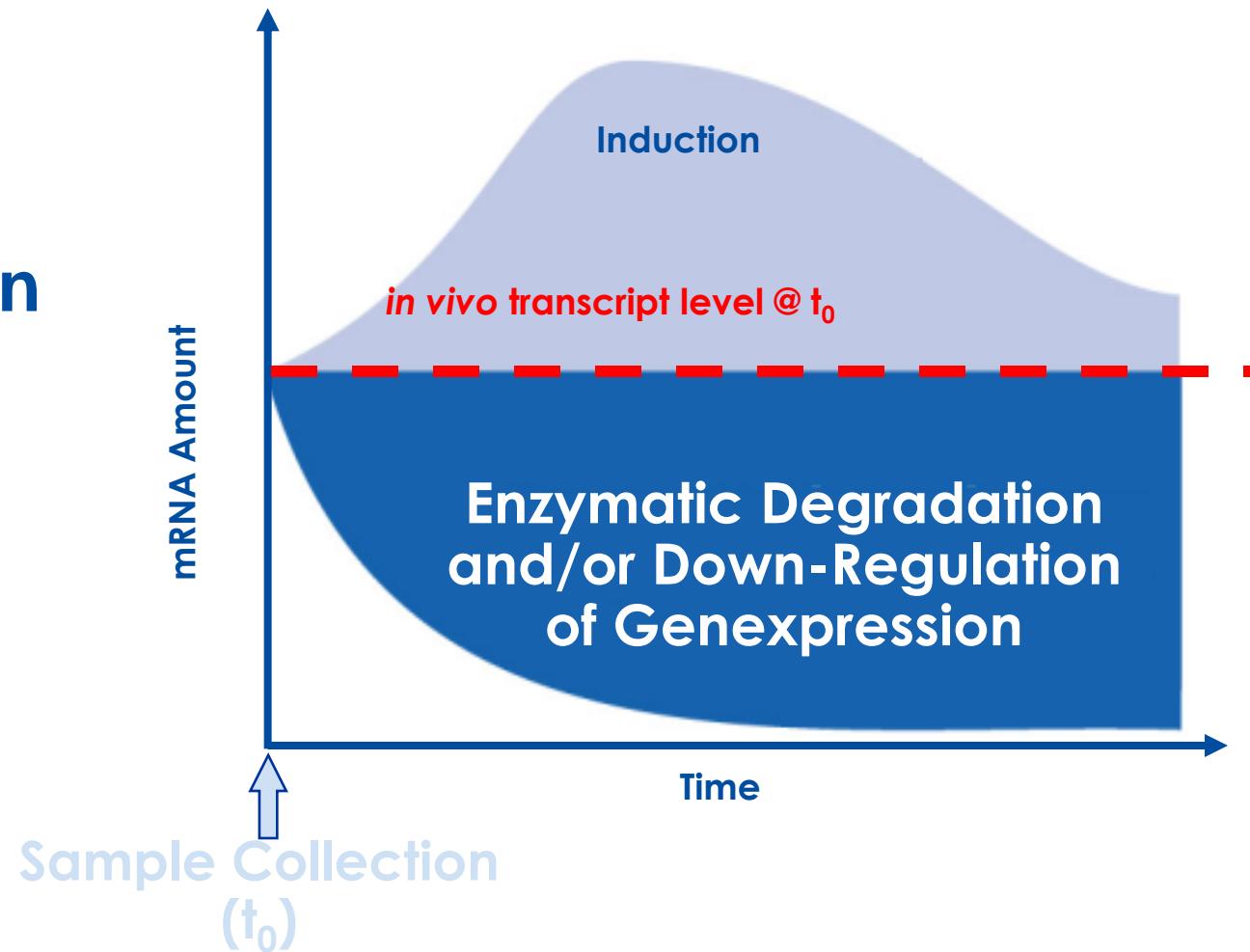
# BENEFITS OF STABILIZATION UTILIZATION

- Direct draw tube with stabilizing reagent
- Stabilization during collection
- Transfer to lab at RT
- Storage at RT, 4°C, -20°C or -70°C



# RNA STABILITY

**Without RNA-Stabilization  
no Stabilization of  
in vivo Genexpression-  
Patterns**



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# TECHNOLOGY FOR DISRUPTION & PURIFICATION



Following sample management, the sample will then have to be disrupted and the molecule (DNA, RNA or protein) purified.

So let's have a look at the challenges that are associated with disruption and purification, as well as QIAGEN's solutions.



# DISRUPTION METHODS

## ROTOR-STATOR HOMOGENIZER

- Simultaneously disrupts and homogenizes animal tissues and cell lysates.

## Mortar and pestle

- Disrupts samples but does not homogenize. Use rotor-stator homogenizer and bead mill for higher yields from animal tissues.
- Use mortar and pestle for plants and filamentous fungi, Do not replace with rotor-stator homogenizer.

## BEAD MILL

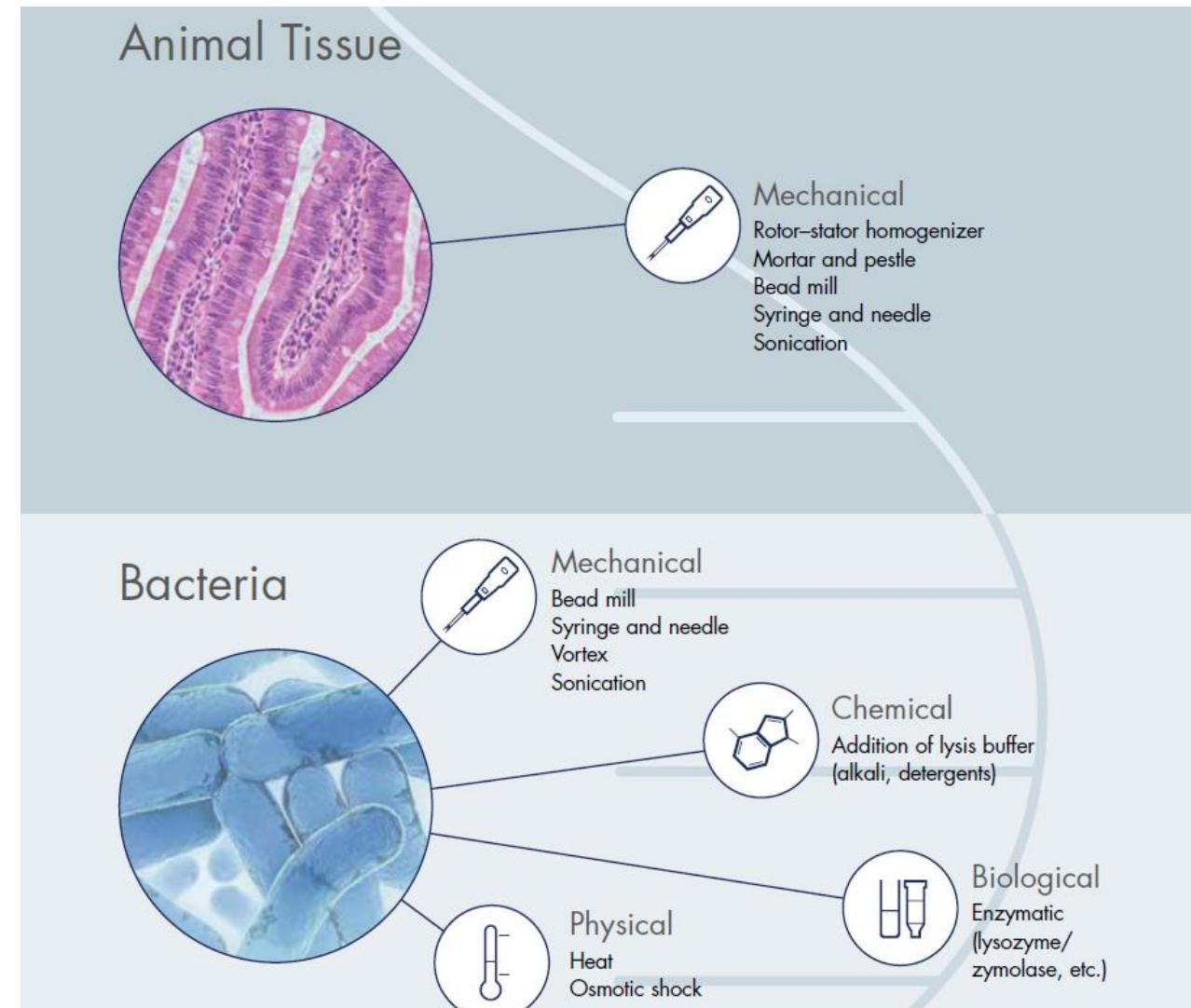
- Simultaneously disrupts and homogenizes; do not replace by vortexing for some bacteria and yeast.

## SYRINGE AND NEEDLE

- Homogenizes viscous cell and tissue lysates.
- Use syringe and needle for higher yields from  $>5 \times 10^8$  bacterial cells.

## VORTEX

- Homogenize by vortexing when processing  $\leq 1 \times 10^5$  mammalian cells.



# DISRUPTION METHODS

## SONICATION

- Suitable for cells, bacteria and finely diced tissue.
- Do not use alone for solid, resilient animal tissues.
- Addition of lysis buffer (alkali, detergents)
- Use in combination with other methods.
- Ideal for cultured animal cell disruption.
- Add after enzymatic digestion of bacteria and yeast.

## ENZYMATIC (lysozyme/zymolase, etc.)

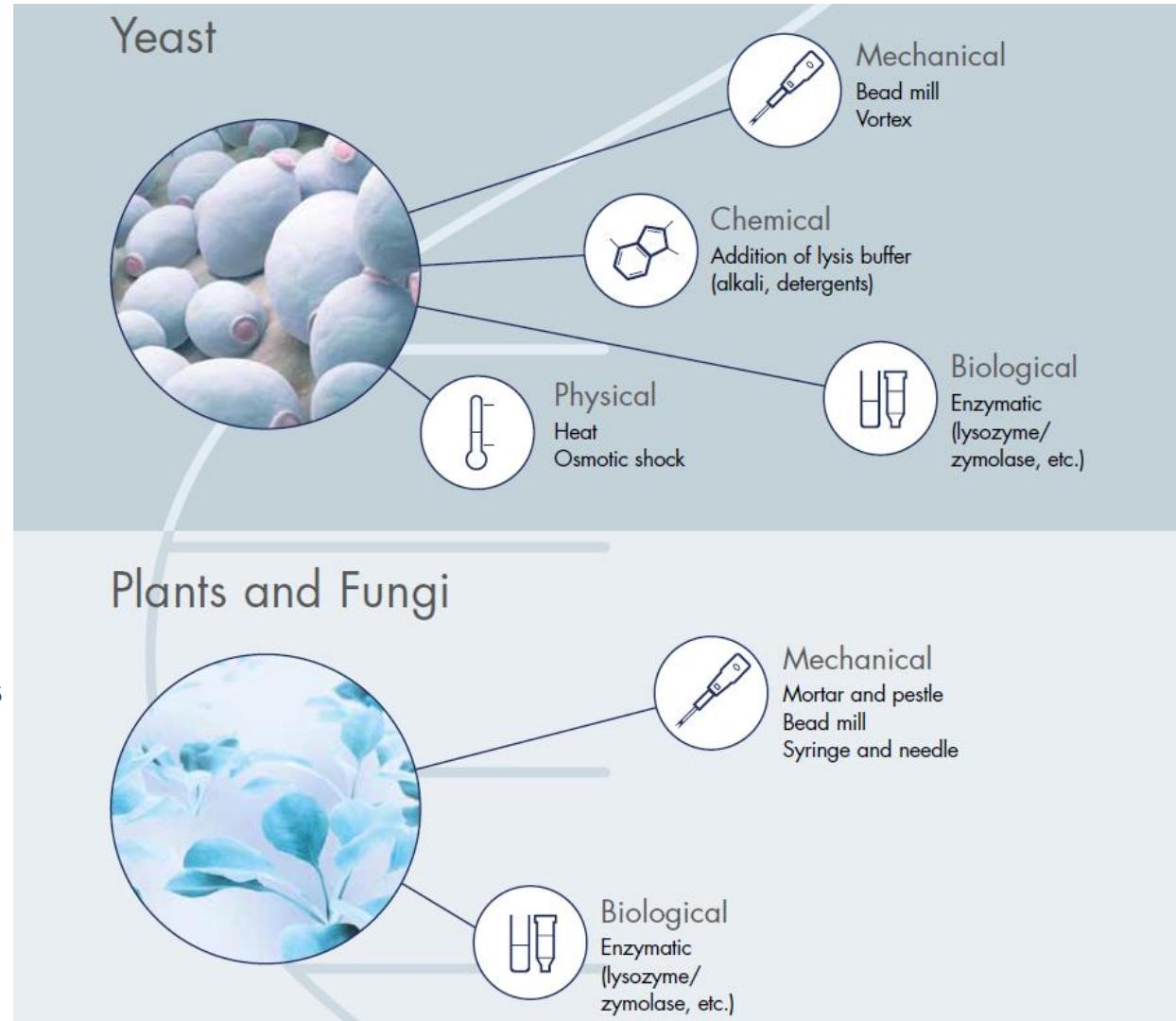
- Bacteria: Lysozyme digestion followed by addition of lysis buffer.
- Yeast: Cell wall digestion (lyticase/zymolase) followed by lysis of spheroplasts.
- Use freshly harvested samples for enzymatic lysis of yeast cells or isolation of cytoplasmic RNA from animal cells.

## HEAT

- Ideal for most viruses. Use in combination with other methods.

## OSMOTIC SHOCK

- Ideal for microbial cell disruption.
- Use in combination with other methods.



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# CHALLENGES



There are several challenges associated with disruption and purification. Maybe you have your own experiences with some of them:

Tough sample

Small amount of sample

Lysis without degradation

Quick and easy processing of samples

Removal of metabolites and enzyme inhibitors

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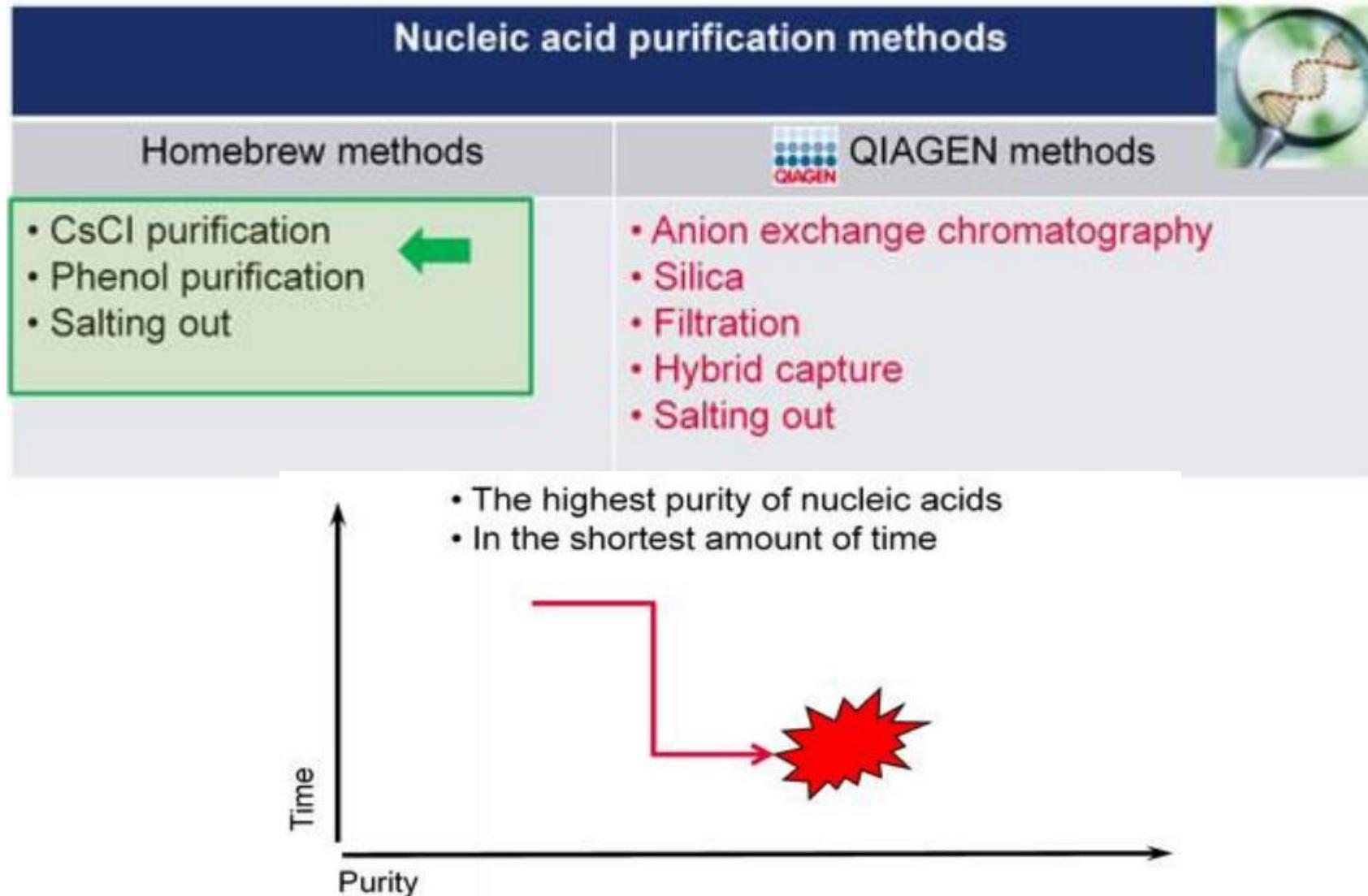
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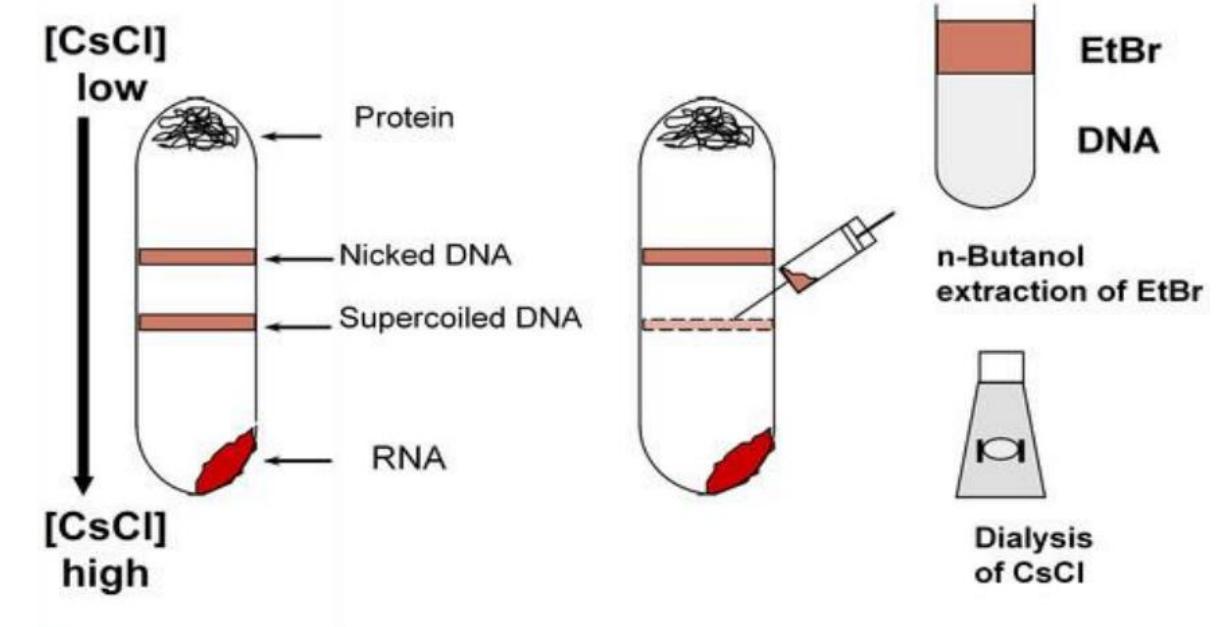
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# Homebrew vs Modern Methods



# Homebrew methods (CsCl purification)

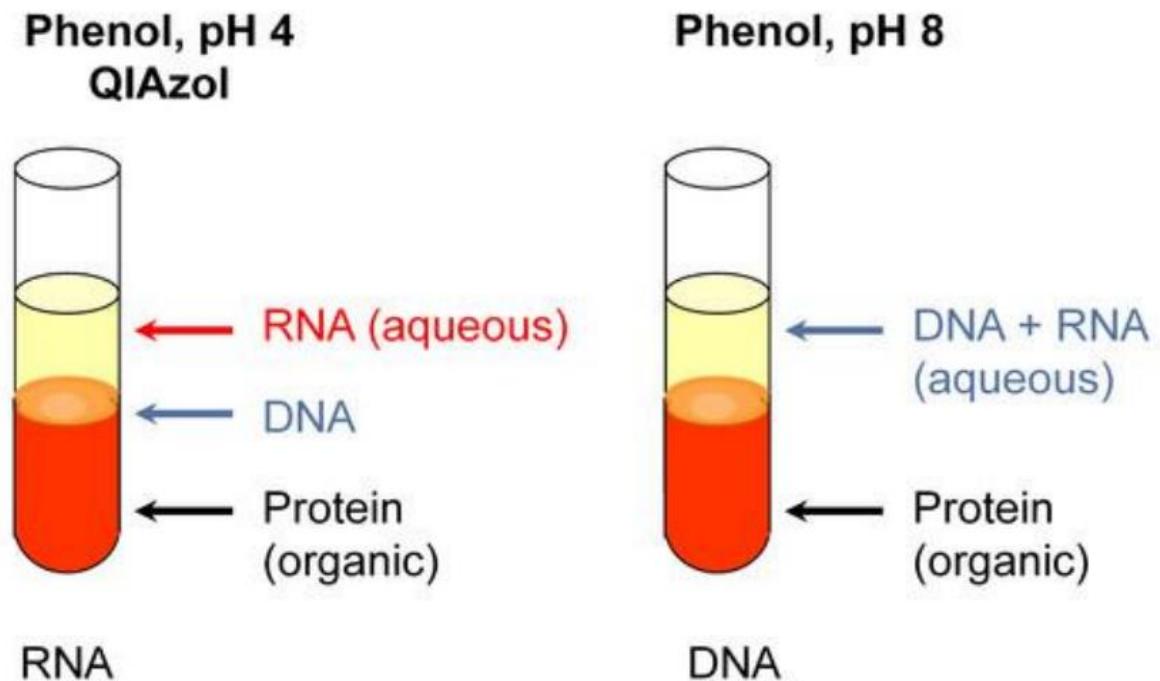
- On the basis of their densities and visualized with ethidium bromide
- Very long procedure
- hypodermic needle utilization
- 2x CsCl gradient purification for higher pure of DNA



# Homebrew methods (Phenol purification)



- Popular methods
- Can separate DNA and RNA by pH change
- Human error prone

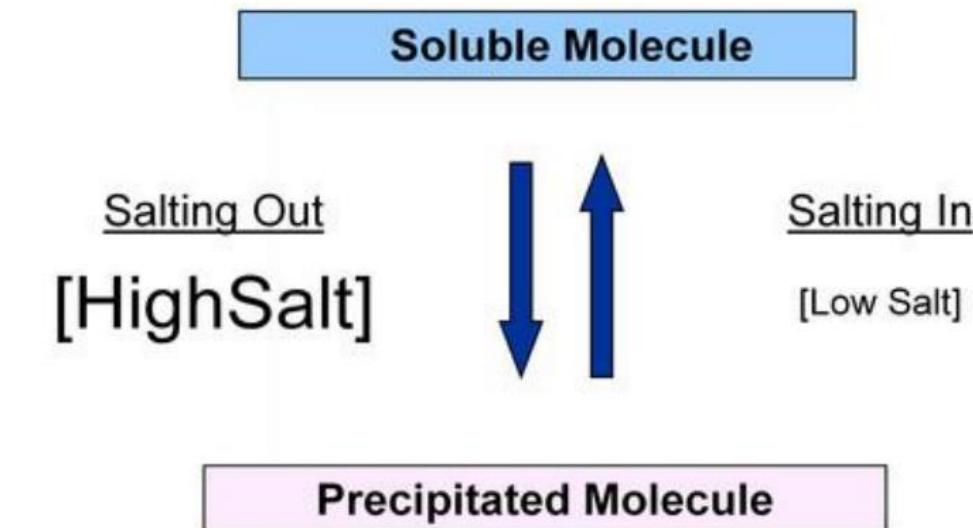


# Homebrew methods (Salting out purification)



- forces the DNA to precipitate out of solution
- separated by centrifugation
- The pellet is washed
- Resuspend with low salt

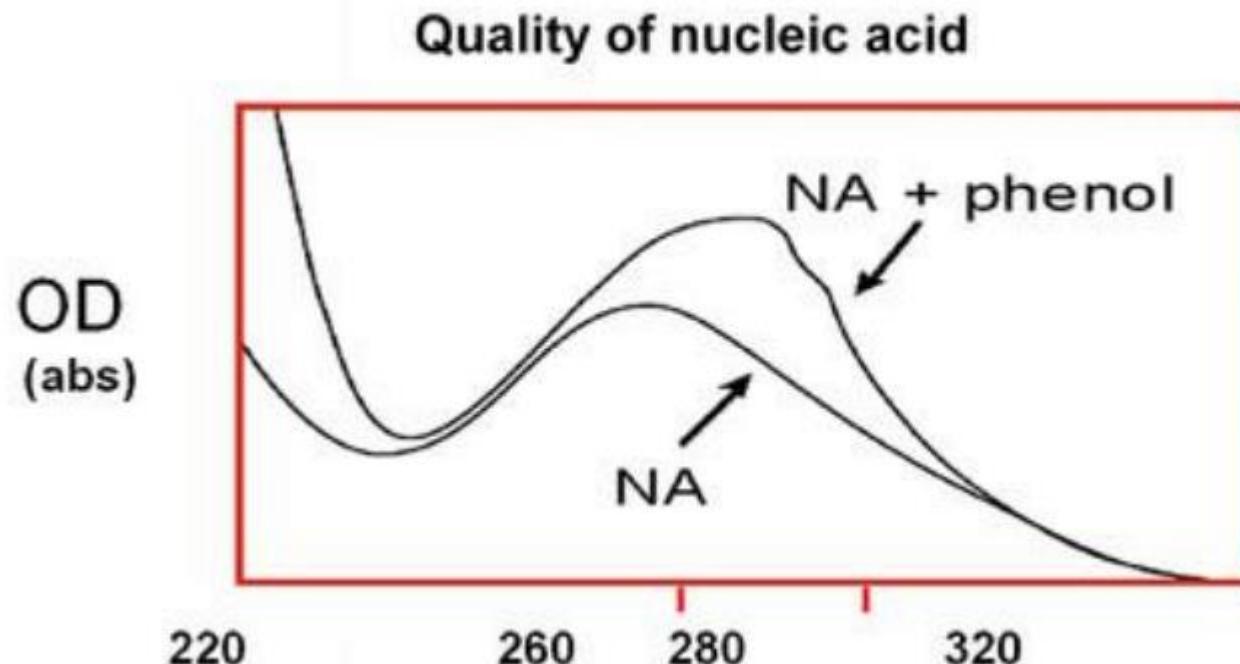
Salt concentration in a solution affects the amount of water available to solubilize a specific molecule



# Disadvantage



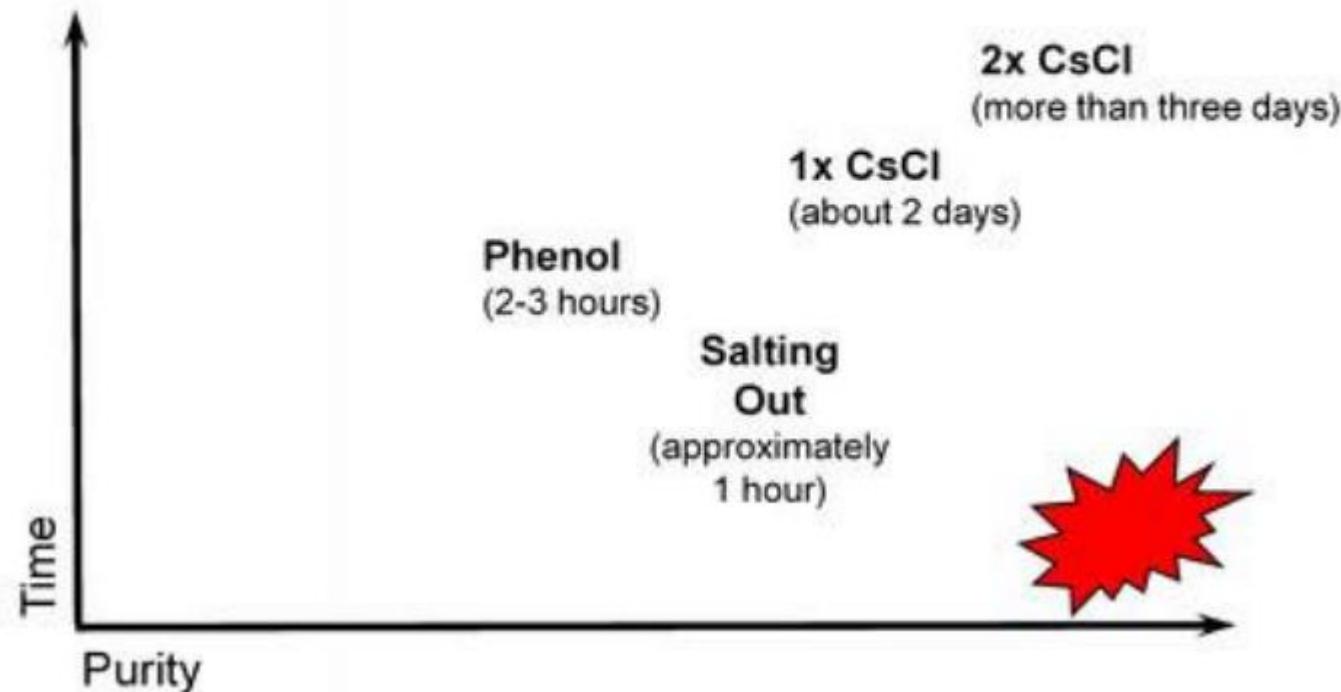
A small amount of phenol contamination has a big impact on your results!



Do you know why? Click on the illustration to get an explanation

# Purity Vs Time

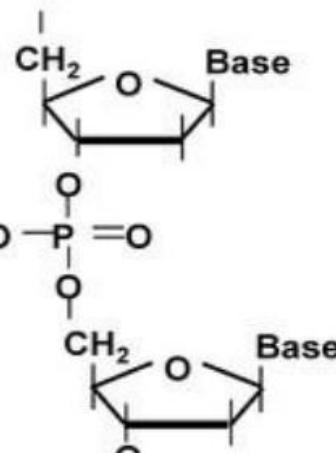
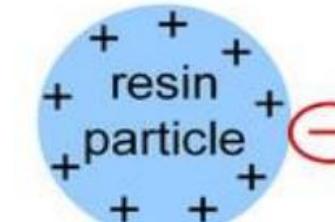
Here you can see how each of these homebrew methods meet the requirements of **highest purity in the shortest amount of time**, two main considerations, when choosing a method.



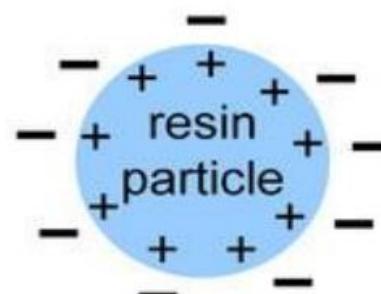
# Modern technology (Anion Exchange)

Conditions for anion-exchange chromatography

■ Low Salt



■ High Salt



**DNA**

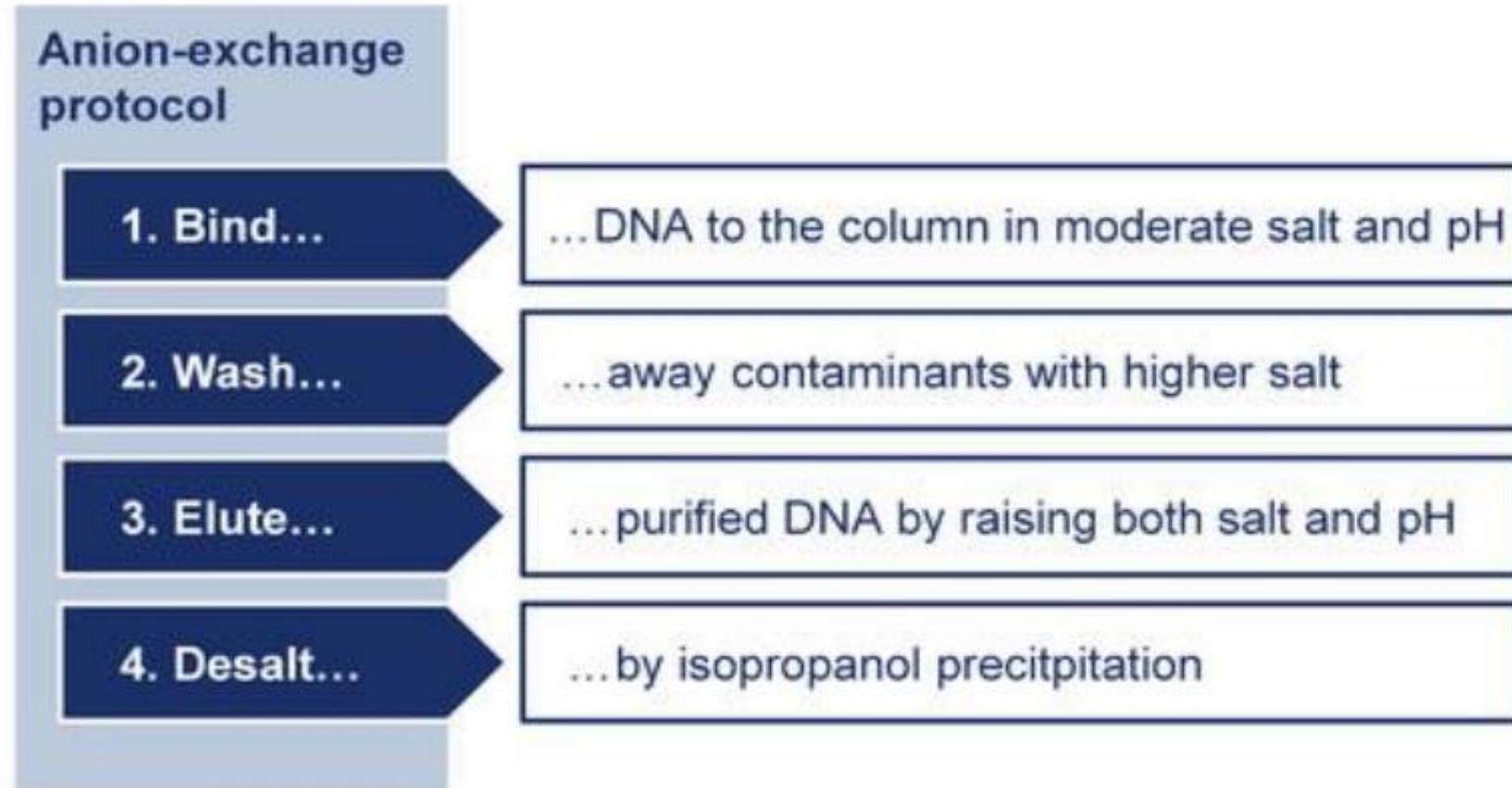
(aqueous)

Elution

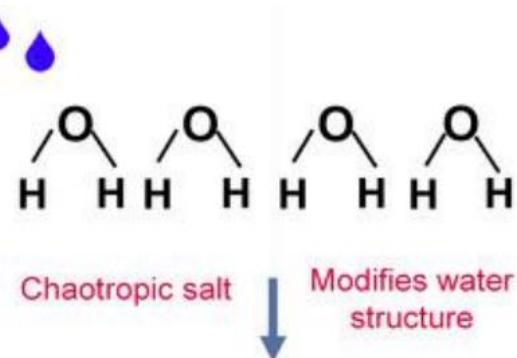
High Salt  
Moderate to High pH

(acid  
rin)

# Modern technology (Anion Exchange)

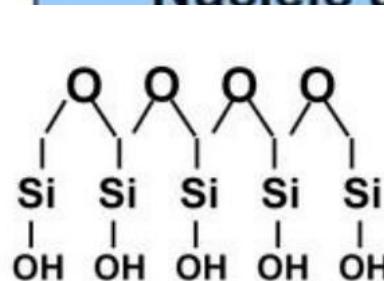
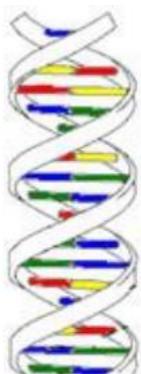


# Modern technology (Silica)



Chaotropic salts are used in the silica purification process. The examples of chaotropic salts that are listed in red are used in QIAGEN silica kits.

- Guanidine isothiocyanate (GITC)
  - Guanidine hydrochloride (GuHCl)
  - Urea
  - Sodium perchlorate ( $\text{NaClO}_4$ )
  - Sodium iodide (NaI)



## Nucleic acid (aqueous)

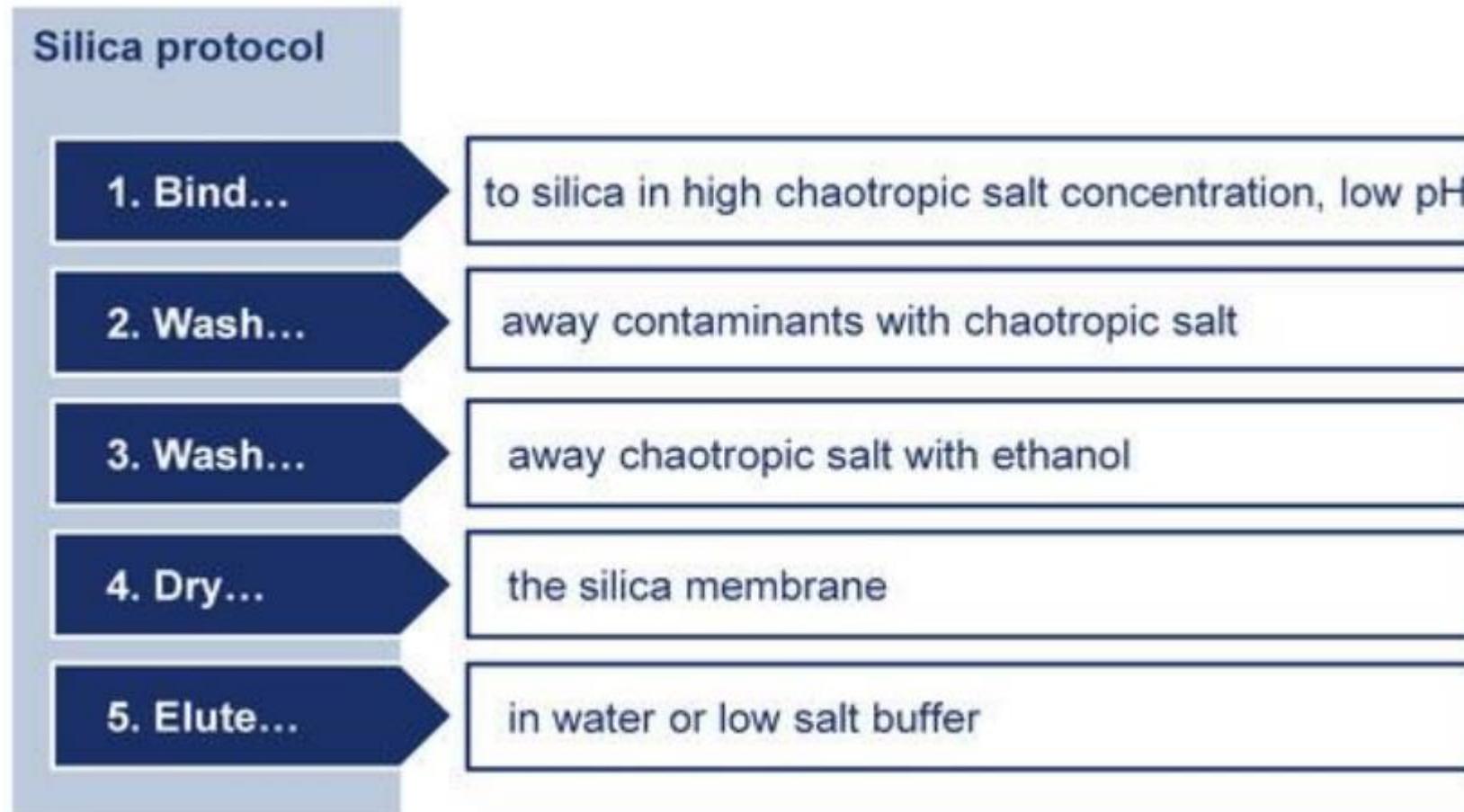
### Elution

No Chaotropic salt  
Low salt  
High pH

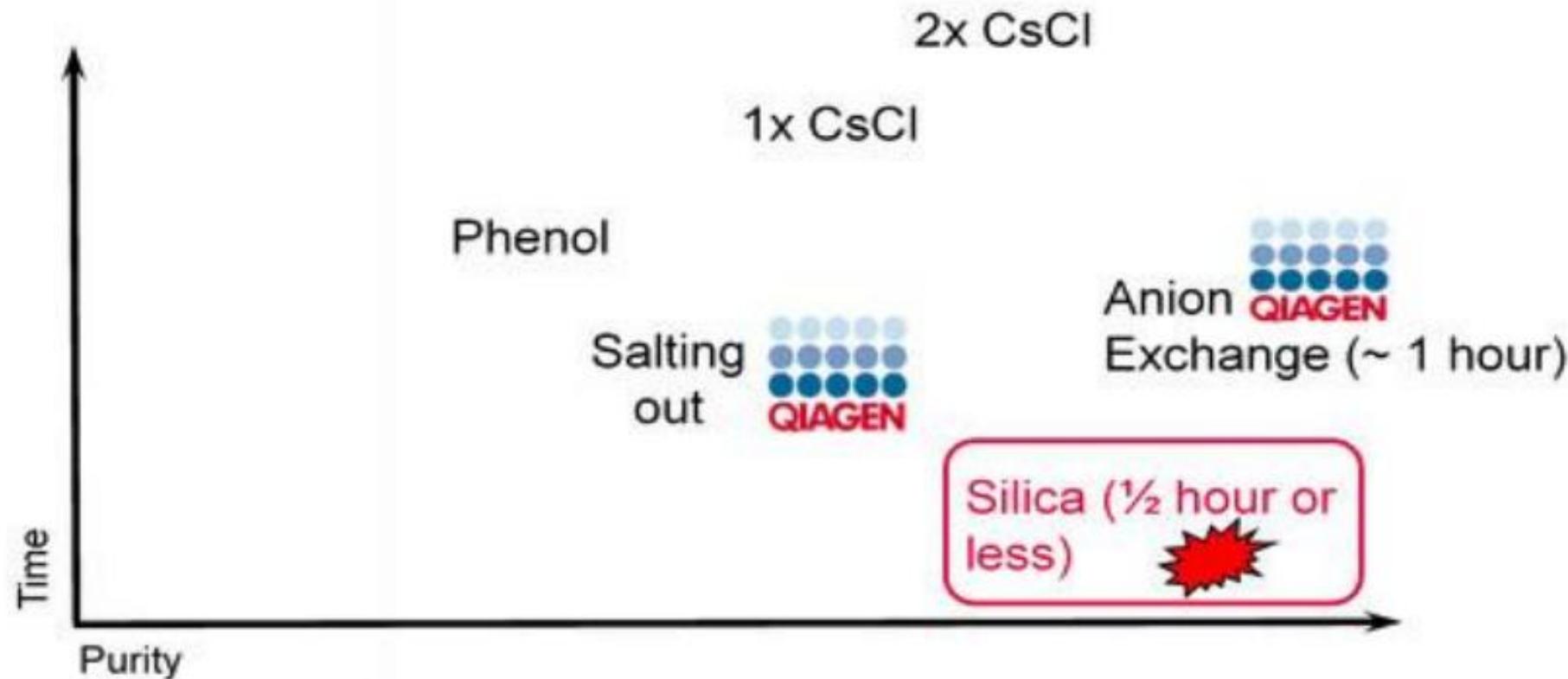


eic acid  
orbated to silica)

# Modern technology (Silica)



# Purity Vs Time



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# Optimizing Extraction for Accurate Results



## PRE-EXTRACTION STEP

- Sample knowledge
- Dedicated kit

## EXTRACTION STEP

- Enhancer compound
- gDNA host depletion
- One sample for separate DNA & RNA isolation

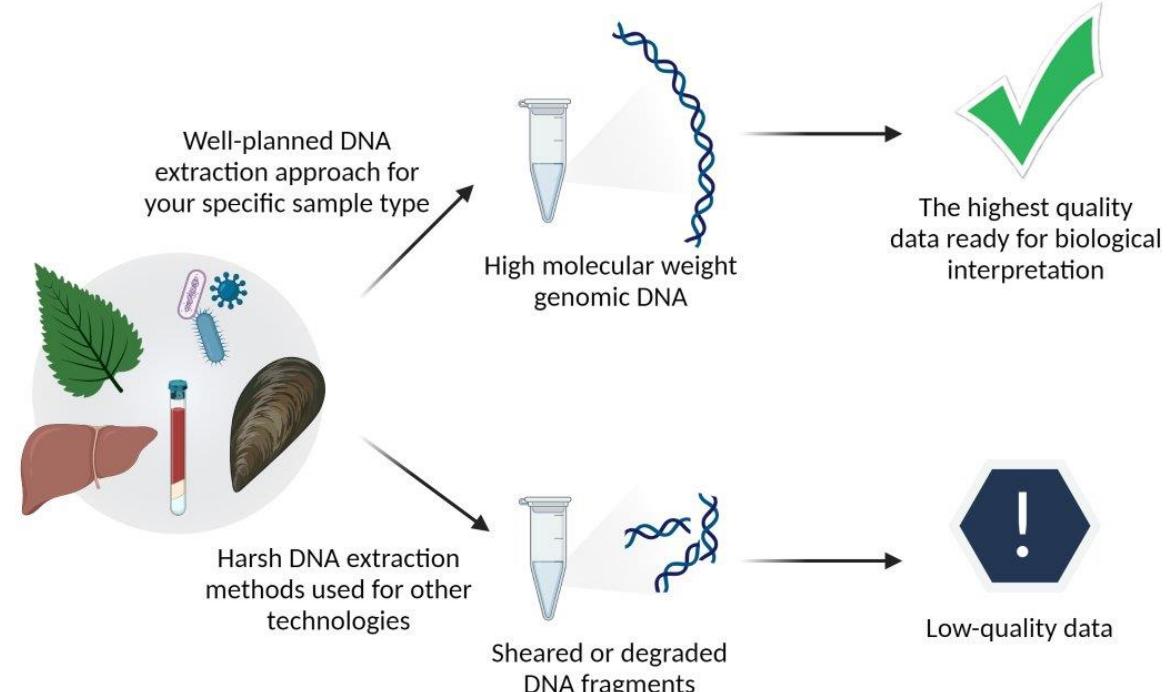
## POST EXTRACTION STEP

- QC step properly

# PRE-EXTRACTION STEP

- Knowing your sample type deeply<sup>a</sup>:
  - ❖ Inhibitor level: **Soil/wastewater/feces** vs **blood/cell-culture/bacteria culture**
  - ❖ Toughness: **tissue/plant/bacteria** vs **virus/human/animal-cell**
  - ❖ DNA/RNA of interest concentration: **cfDNA/ctDNA** vs **gDNA**
  - ❖ Sample management
- Dedicated Kit
  - ❖ Commercial kit: **magnetic beads/spin based column**
  - ❖ QIAGEN: 80 dedicated kit

## DNA Extraction for Optimal Sequencing Results

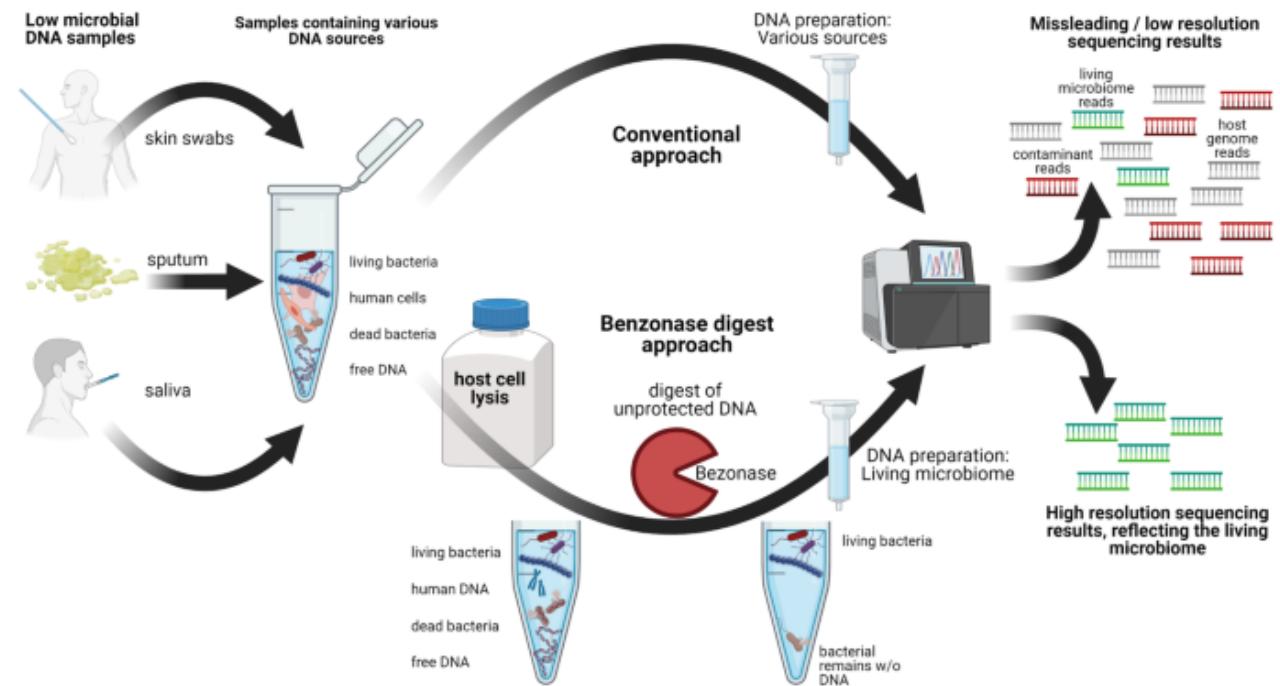


a. Shaffer JP, Marotz C, Belda-Ferre P, Martino C, Wandro S, Estaki M, Salido RA, Carpenter CS, Zaramela LS, Minich JJ, Bryant M, Sanders K, Fraraccio S, Ackermann G, Humphrey G, Swafford AD, Miller-Montgomery S, Knight R. A comparison of DNA/RNA extraction protocols for high-throughput sequencing of microbial communities. *Biotechniques*. 2021 Mar;70(3):149-159. doi: 10.2144/btn-2020-0153. Epub 2021 Jan 29. Erratum in: *Biotechniques*. 2024 Mar;76(3):119. doi: 10.2144/btn-2020-0153c1. PMID: 33512248; PMCID: PMC7931620.

# EXTRACTION STEP

## gDNA HOST DEPLETION<sup>b</sup>

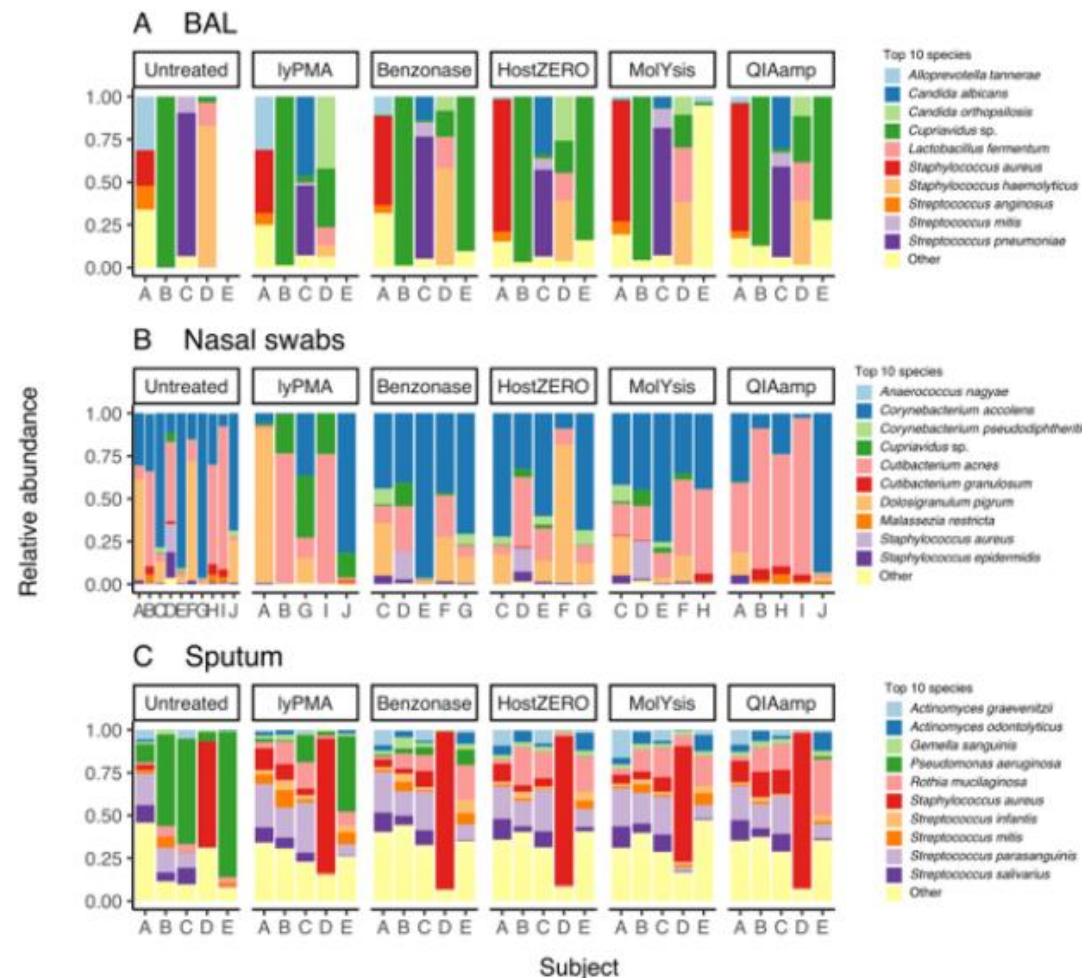
- **Buffer lysis for host cell:** strong enough for host-cell, weak enough for microbe cell
- **DNA Host removal**
  - DNase with high effective activity:** Benzonase, MoldNase,
  - microbial selection enzyme**
  - Capture methylated host DNA**
  - Capture non-methylated CpG dinucleotides**
- **Each type sample has own effective host depletion methods**



<sup>b</sup>) Heravi FS, Zakrzewski M, Vickery K, Hu H. Host DNA depletion efficiency of microbiome DNA enrichment methods in infected tissue samples. *J Microbiol Methods*. 2020 Mar;170:105856. doi: 10.1016/j.mimet.2020.105856. Epub 2020 Jan 30. PMID: 32007505.

# Why is DNA host depletion so important???

- Increased reads number microbial DNA
- Cost efficiency
- Avoid false alpha diversity result
- Avoid false beta diversity result

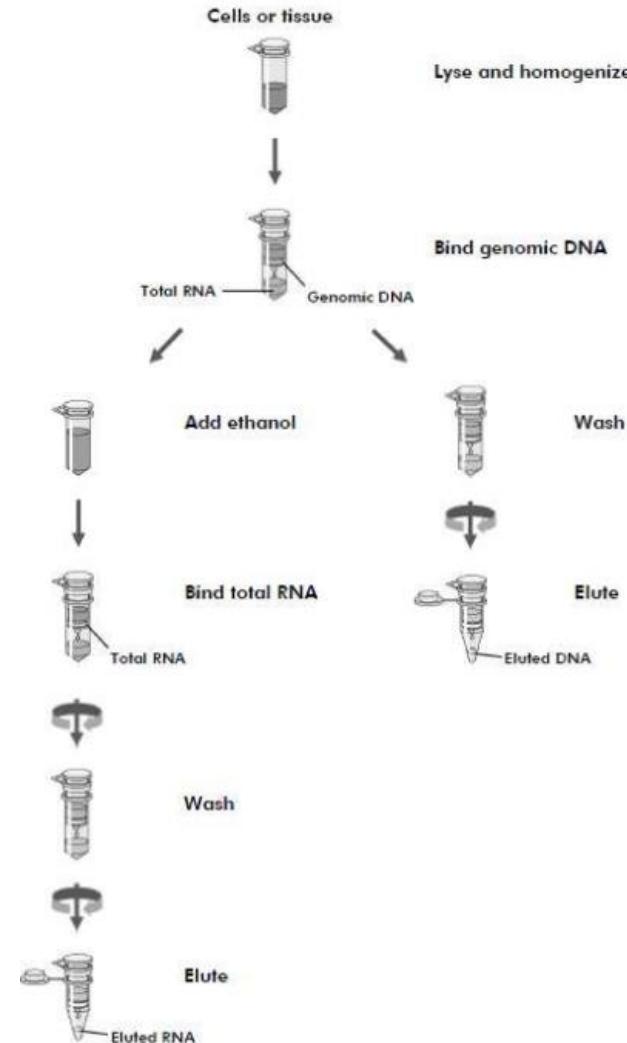


Heravi FS, Zakrzewski M, Vickery K, Hu H. Host DNA depletion efficiency of microbiome DNA enrichment methods in infected tissue samples. *J Microbiol Methods*. 2020 Mar;170:105856. doi: 10.1016/j.mimet.2020.105856. Epub 2020 Jan 30. PMID: 32007505

# EXTRACTION STEP

## MULTIMODAL (DNA&RNA)

- Generate DNA&RNA from one sample
- Reduce bias for association genotype & phenotype study
- Extraction kit: Allprep DNA/RNA mini kit
- NGS kit: QIAseq Multimodal panel



## MinElute® Kits

- MinElute Gel Extraction Kit
- MinElute PCR Purification Kit
- MinElute Reaction Cleanup Kit

## QIAquick® Kits

- QIAquick Gel Extraction Kit
- QIAquick Nucleotide Removal Kit
- QIAquick PCR Purification Kit

## Power Kits

- DNeasy PowerBiofilm® Kit
- DNeasy PowerClean® Pro Cleanup Kit
- QIAamp PowerFecal® Pro DNA Kit
- QIAamp PowerFecal® Pro DNA QIAcube® Kit
- DNeasy PowerLyzer® PowerSoil® Kit
- DNeasy PowerSoil® Kit
- DNeasy PowerSoil® Pro Kit
- DNeasy PowerSoil® Pro QIAcube® Kit
- DNeasy PowerWater® Kit
- RNeasy PowerFecal® Pro Kit
- RNeasy PowerMicrobiome™ Kit
- AllPrep PowerViral DNA/RNA Kit

## DNeasy® Kits

- DNeasy Blood & Tissue Kit
- DNeasy Blood & Tissue QIAcube® Kit
- DNeasy mericon™ Food Kit
- DNeasy Plant Mini Kit

## DNeasy® Kits

- DNeasy Plant Pro Kit
- DNeasy ultra-clean® Microbial Kit

## QIAamp® Kits

- QIAamp Circulating Nucleic Acid Kit
- QIAamp ccfDNA/RNA Kit
- QIAamp DNA Blood Mini Kit
- QIAamp DNA Blood Mini QIAcube® Kit
- QIAamp DNA FFPE Advanced UNG Kit
- QIAamp DNA FFPE Tissue Kit
- QIAamp DNA FFPE Advanced Kit
- QIAamp DNA Investigator Kit
- QIAamp DNA Mini Kit
- QIAamp DNA Mini QIAcube® Kit
- QIAamp Fast DNA Tissue Kit
- QIAamp Fast DNA Stool Mini Kit
- QIAamp MinElute ccfDNA Mini Kit
- QIAamp MinElute® Media Kit
- QIAamp MinElute® Virus Spin Kit
- QIAamp RNA Blood Mini Kit
- QIAamp UCP DNA Micro Kit
- QIAamp Viral RNA Mini Kit
- QIAamp Viral RNA Mini QIAcube® Kit

## RNeasy® Kits

- RNeasy FFPE Kit
- RNeasy Fibrous Tissue Mini Kit
- RNeasy Lipid Tissue Mini Kit
- RNeasy Micro Kit
- RNeasy Mini Kit

## RNeasy® Kits

- RNeasy Mini QIAcube® Kit
- RNeasy MinElute® Cleanup Kit
- RNeasy Plant Mini Kit
- RNeasy Plus Micro Kit
- RNeasy Plus Mini Kits
- RNeasy Plus Universal Mini Kit
- RNeasy Protect Bacteria Mini Kit
- RNeasy Protect Animal Blood Kit
- RNeasy Pure mRNA Bead Kit
- RNeasy UCP Micro Kit
- miRNeasy FFPE Kit
- miRNeasy Micro Kit
- miRNeasy Mini Kit
- miRNeasy Serum/Plasma Kit
- miRNeasy Serum/Plasma Advanced Kit
- exoRNeasy Midi Kit
- miRNeasy Tissue/Cells Advanced Mini Kit

## QIAprep® Kit

- QIAprep Spin Miniprep Kit

## AllPrep® Kits

- AllPrep DNA/RNA FFPE Kit
- AllPrep DNA/RNA Micro Kit
- AllPrep DNA/RNA Mini Kit
- AllPrep DNA/RNA/miRNA Universal Kit

## GeneRead™ Kits

- GeneRead DNA Library Q
- GeneRead Size Selection Kit

## PAXgene® Kits

- PAXgene Blood miRNA Kit
- PAXgene Tissue DNA Kit
- PAXgene Tissue RNA/miRNA Kit

## Bisulfite Conversion Kits

- EpiTect Bisulfite Kit
- EpiTect Fast DNA Bisulfite
- EpiTect Plus DNA Bisulfite Kit

## Protein Kits

- Ni-NTA Spin Kit

## QIAwave Kits

- QIAwave RNA Mini Kit
- QIAwave DNA Blood & Tissue Kit
- QIAwave Plasmid Mini Kit
- QIAwave DNA/RNA Mini Kit
- QIAwave RNA Mini Kit
- QIAwave RNA Plus Mini Kit

**Dedicated QIAcube Kits:** include optimised buffer volumes and Rotor Adapters (pre-loaded with spin columns and elution tubes)

**QIAwave Kits:** include spin columns (not preassembled with waste tubes) packed in a bag instead of blisters; for a more convenient run setup

# Enhancer (One of solutions for Small DNA target)



There's one **particular challenge** with nucleic acid purification: researchers often want to **recover** nucleic acid from very small samples with **few target molecules**.



QIAGEN's solution is *carrier RNA*:



- Must be present during the binding step
- Enhances binding of nucleic acid to the silica membrane
- Results in improved recovery of nucleic acids

# QUALITY CONTROL (Post-extraction)



## Purity

- Usually judged by OD ratio
- Absence of contaminants
- Absence of gDNA
- Stability of eluates

## Integrity – no degradation

- Usually judged by (capillary) gel electrophoresis (e.g., agarose gels, QIAxcel)
- 3'-5' ratio

## $A_{260}/A_{280}$ ratio

- Lower ratios are commonly associated with protein contamination but sensitivity is rather low
- $A_{260}/A_{280}$  ratio is influenced considerably by pH

		Nanodrop	Qubit
Purity	Measurement method	Use of wavelength	Use of specific binding fluorescence
Integrity – no degradation	Accuracy of DNA quantification	A little low	High
	Measurable concentration value	2 ng/mL or more recommended	100 pg/mL or more (based on dsDNA BR)
	Purity measurement	O	X
	Reagent consumption	X	O



- Guanidine HCl (GuHCl) absorbs at ~230 nm
- Guanidine thiocyanate (GITC) also absorbs at ~230 nm at moderate or low concentrations
  - At very high concentrations, GITC absorbs at 260 nm

# Sample To Insight



## 1 Part 1: OVERVIEW

→ Research workflow

## 2 Part 2: SAMPLE MANAGEMENT

→ Element Of Samples Management

→ Biomolecule Stability

→ Transport & Storage Of Sample Types

## 3 Part 3: DISRUPTION

## 4 Part 4: PURIFICATION

→ Challenges

→ Homebrews vs Modern Technologies

→ Optimizing Extraction for Accurate Results

## 5 Part 5: QIAGEN SOLUTION

## 6 Part 6: NGS library construction

# All-in-one sets tailored for microbiome research

## Microbiome WGS SeqSets



### DNA Extraction

**QIAamp® PowerFecal® Pro  
WGS SeqSet**

**QIAamp PowerFecal Pro  
DNA**

- Stool

OR

**DNeasy® PowerSoil® Pro  
WGS SeqSet**

**DNeasy PowerSoil Pro**

- Soil



### Whole Genome Sequencing (WGS)

**QIAseq® FX DNA Library  
Kit**

- All-enzymatic library prep for WGS

**QIAseq Normalizer Kit**

- Rapid library normalization for high-throughput sequencing

**QIAseq Beads**



### Bioinformatic Analysis

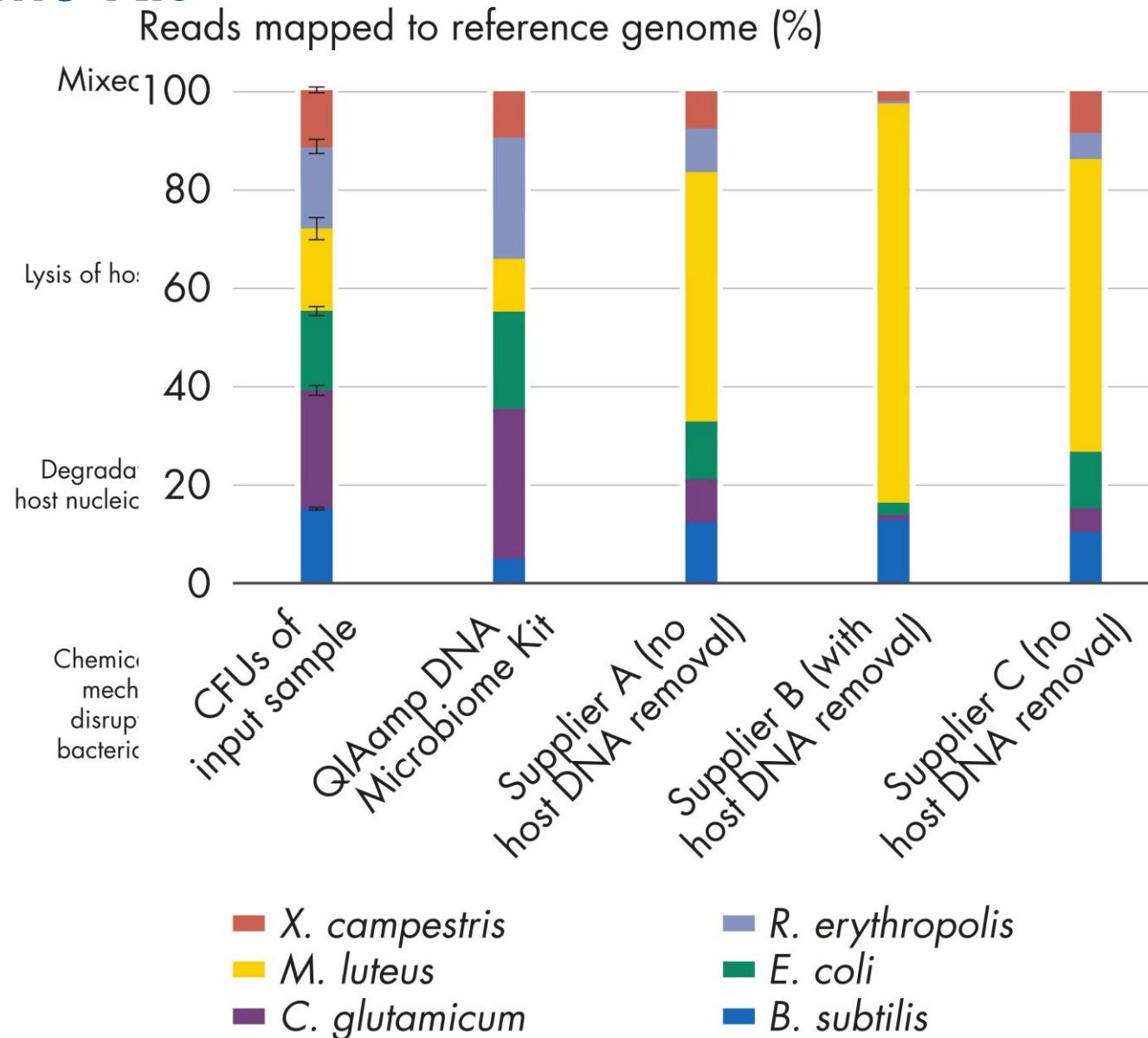
**Microbial Analysis Portal  
(MAP)**

- Comprehensive metagenomic analysis

# QIAamp DNA Microbiome Kit



- **buffer AHL: lysis buffer for Host Cell**
- **Banzonase : DNase to eliminate host Cell**



# PowerProtect DNA/RNA

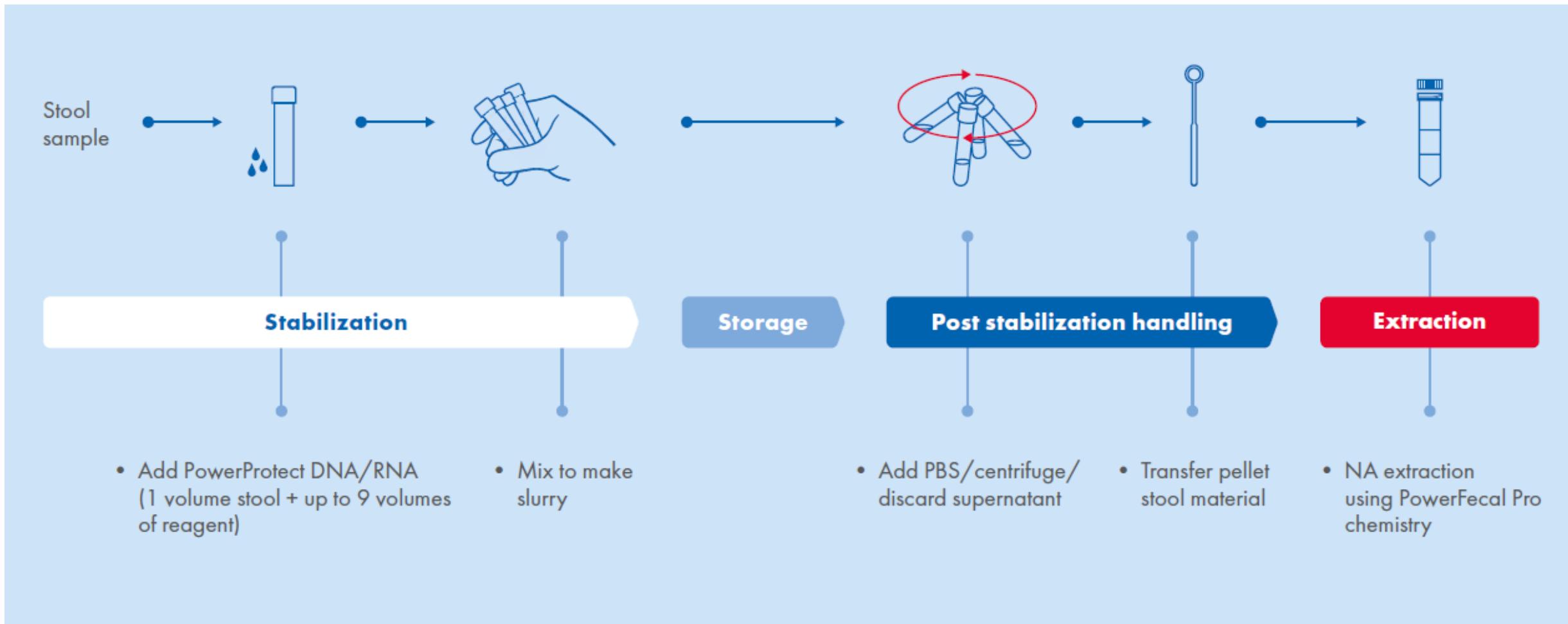


- Preserves bacterial DNA and RNA in stool samples after collection
- Penetrates cells and halts biological activity
- Maintains the microbial community and functional profile during transport and storage

	<b>at 4°C - 25°C</b>	<b>elevated temperature (35°C)</b>	<b>frozen at -30 to -15°C or -90 to -65°C</b>
<b>DNA</b>	indefinite	14 days	indefinite
<b>RNA</b>	at least 2months	3 days	indefinite

It is not recommended to store the stabilized samples at higher temperatures for long periods of time – in particular for RNA. Shipping time is regarded as uncritical

# Stabilization workflow using the PowerProtect DNA/RNA



# Sample To Insight



## 1 Part 1: OVERVIEW

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## 3 Part 3: DISRUPTION

## 4 Part 4: PURIFICATION

→ Challenges

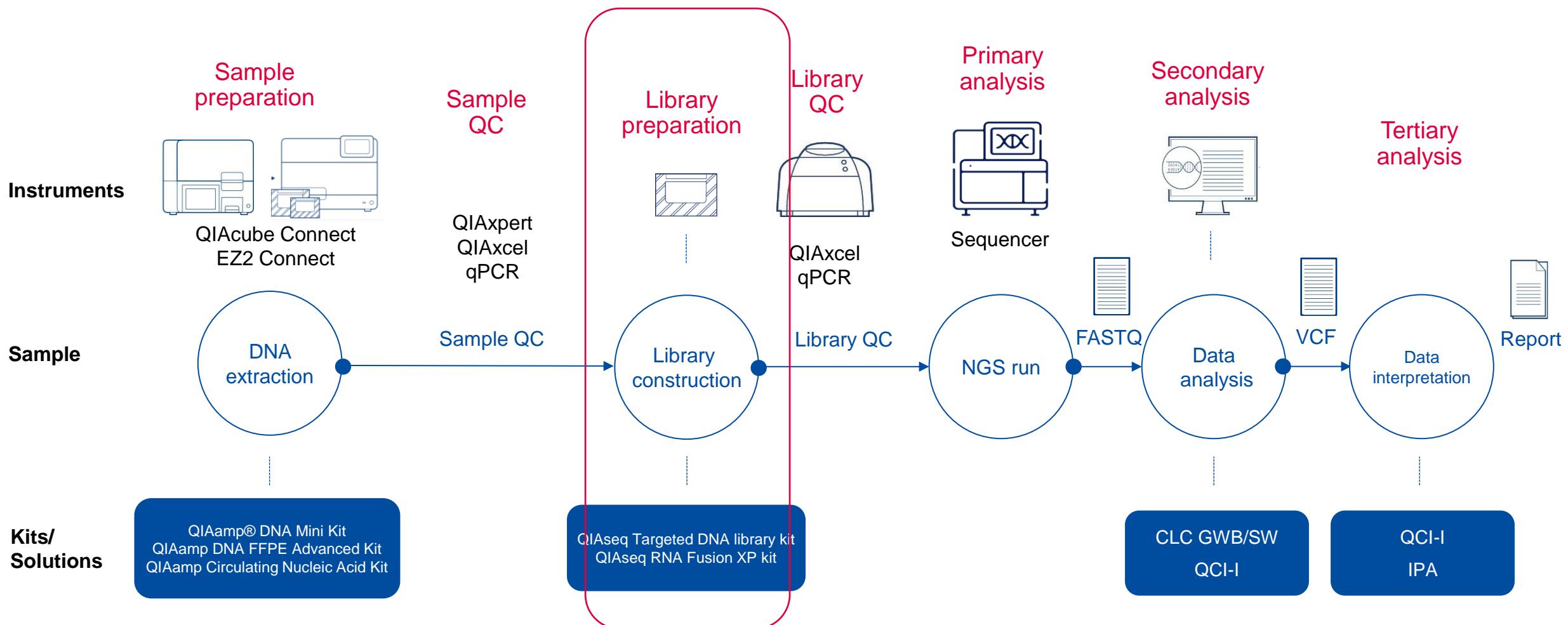
→ Homebrews vs Modern Technologies

→ Optimizing Extraction for Accurate Results

## 5 Part 5: QIAGEN SOLUTION

## 6 Part 6: NGS library construction

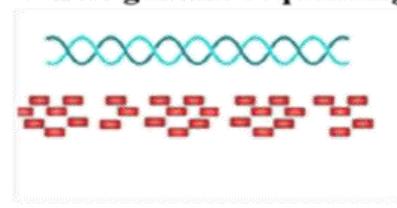
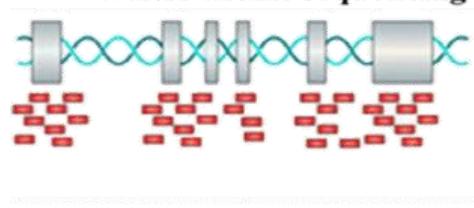
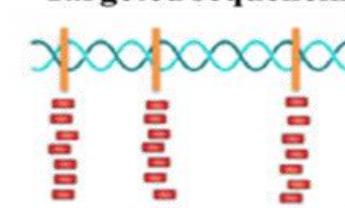
# QIAseq targeted DNA workflow: Sample to Insight



## General structure of library fragments

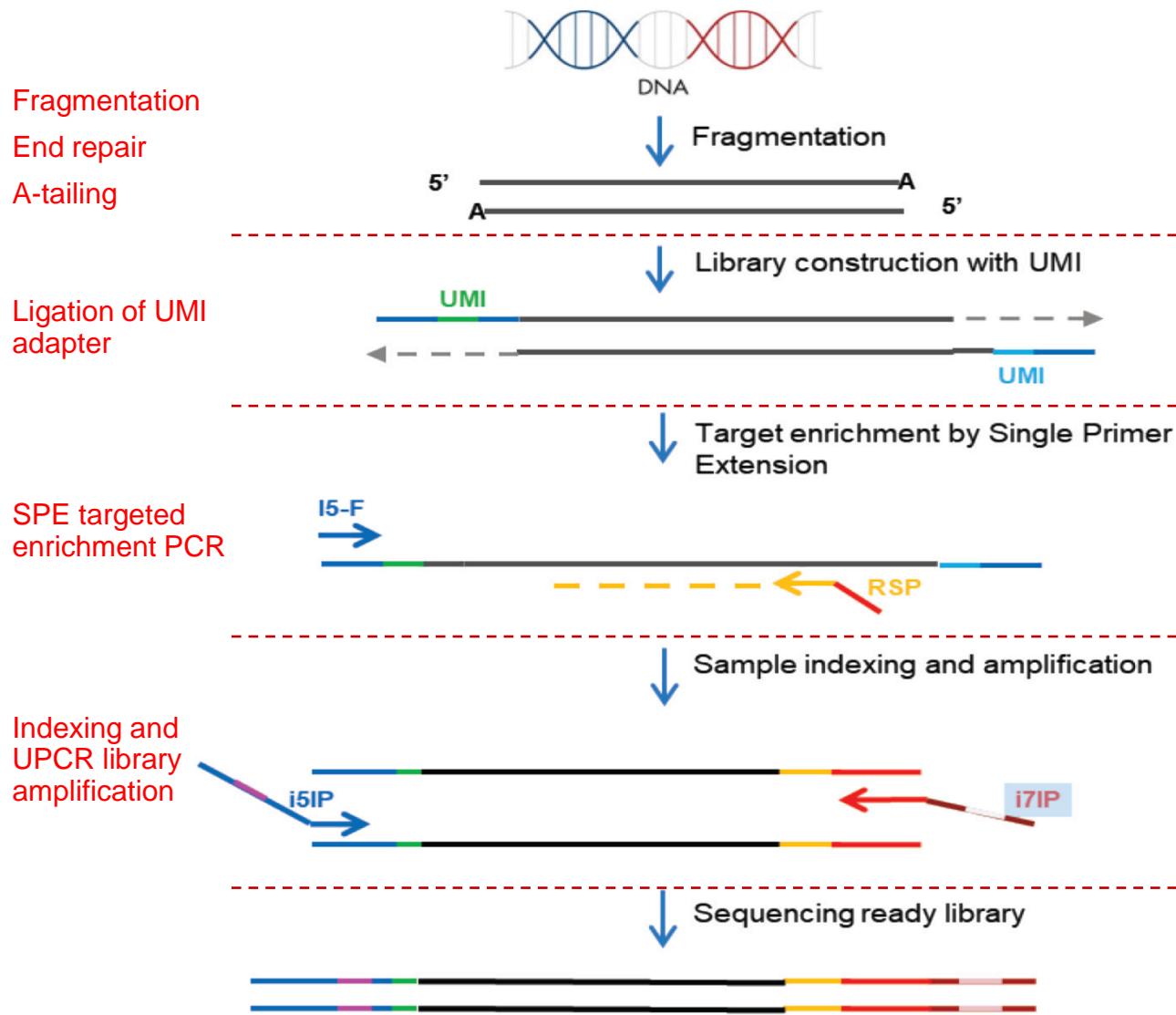


- 1 and 6: sequences used to attach libraries to a solid surface for clonal amplification and sequencing
- 2 and 4: sequences used by sequencing primers to initiate sequencing
- 3: amplicons  generated in workflow step 2 (representing EGFR and KRAS)
- 5: Barcodes to allow multiplexing of samples (running many samples simultaneously in the same sequencing run)

	<b>Whole genome sequencing</b> 	<b>Whole exome sequencing</b> 	<b>Targeted sequencing</b> 
Detection Area	Whole Introns and exons	Whole exons	Specific genes / specific exons / Hotspot
Coverage Depth	+	++	+++
Genome Coverage	+++	++	+
Cost per sample	+++	++	+

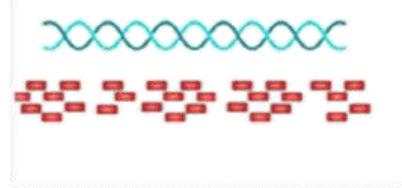
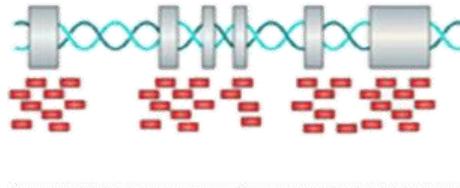
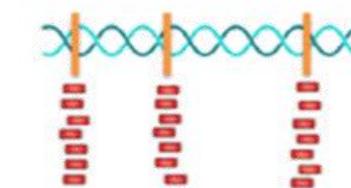
DOI:[10.32604/biocell.2019.08180](https://doi.org/10.32604/biocell.2019.08180)

# Library construction workflow (Targeted Sequencing)



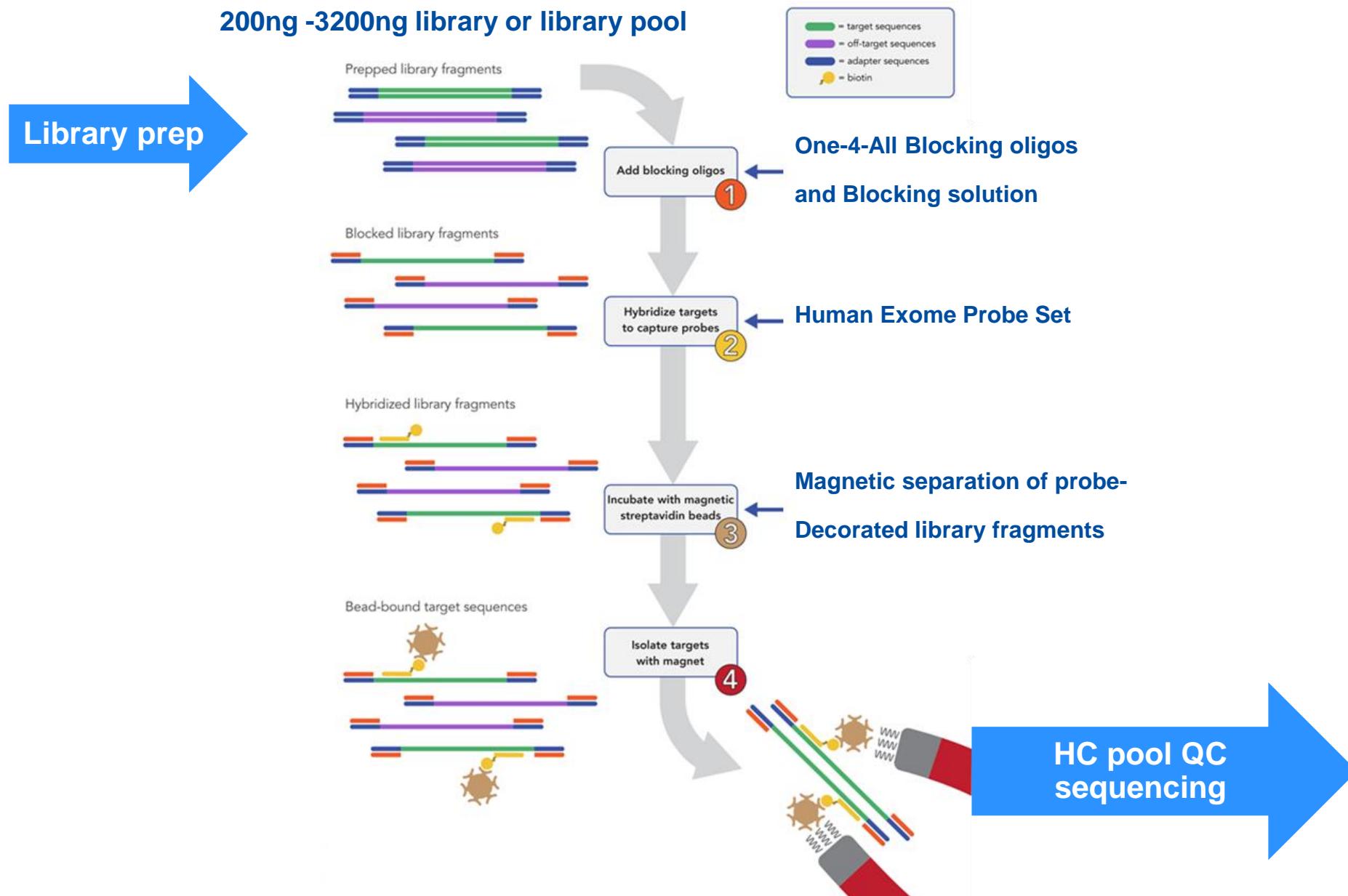
## WGS, WES, targeted sequencing:



	Whole genome sequencing 	Whole exome sequencing  HYB CAPTURE	Targeted sequencing 
Detection Area	Whole Introns and exons	Whole exons	Specific genes / specific exons / Hotspot
Coverage Depth	+	++	+++
Genome Coverage	+++	++	+
Cost per sample	+++	++	+

DOI:[10.32604/biocell.2019.08180](https://doi.org/10.32604/biocell.2019.08180)

# Hybridization / capture / wash



# THANK YOU

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