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The do´s and don`ts of real time RT-PCR as a tool in fish diagnostics

Evaluating important parameters, pitfalls and result bias

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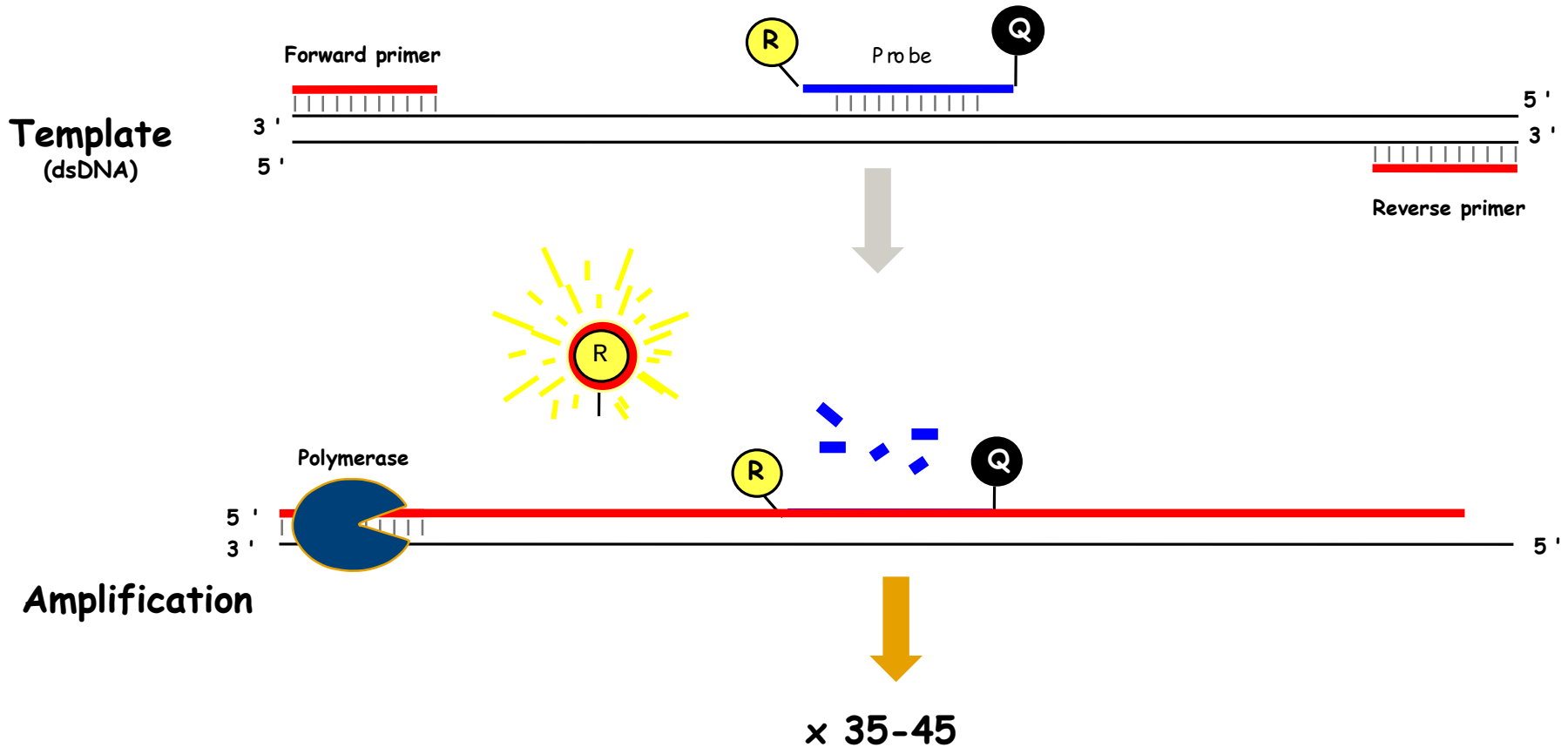
Real time RT-PCR TaqMan assay principle



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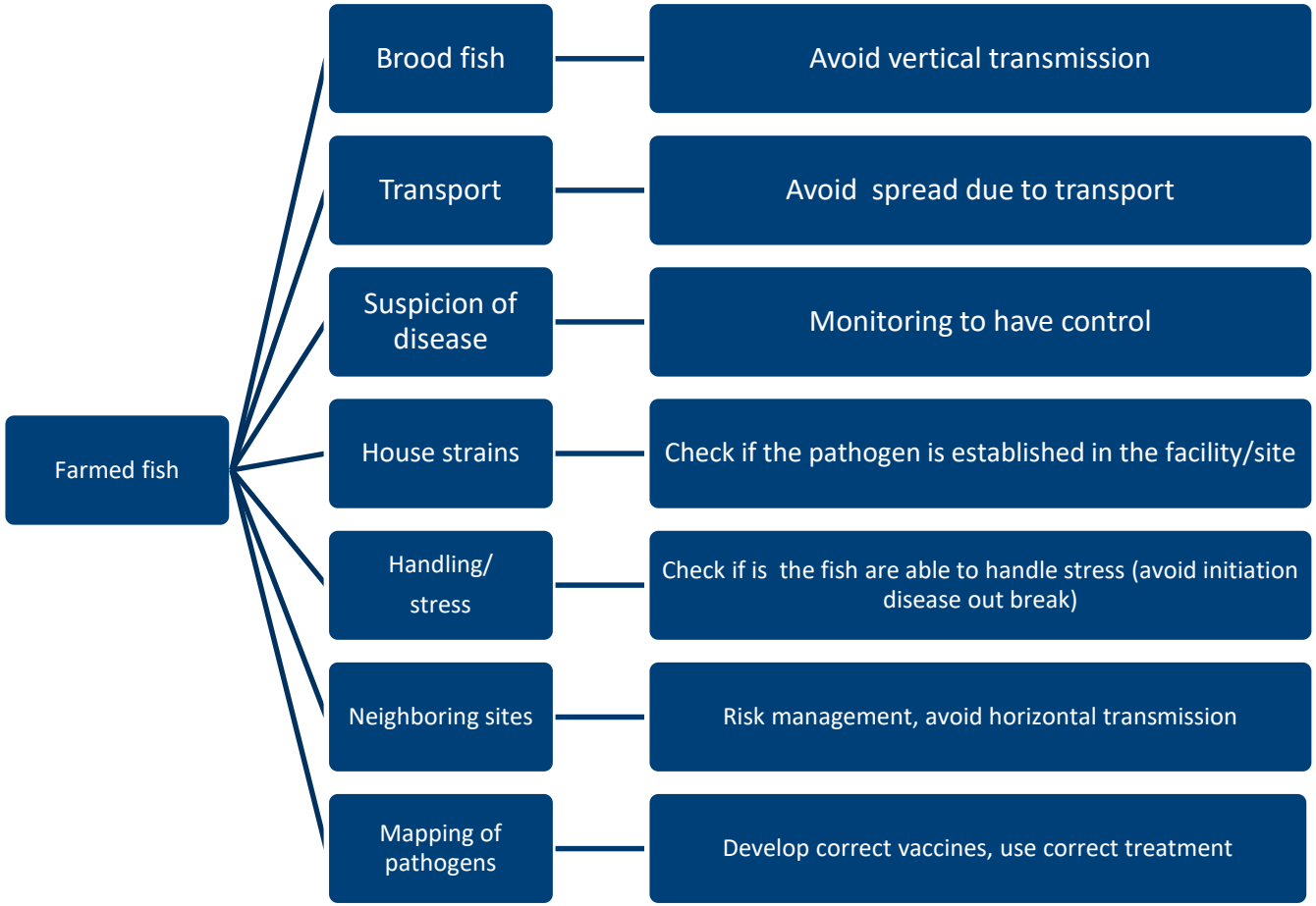
Use of real time RT-PCR in preventive fish health



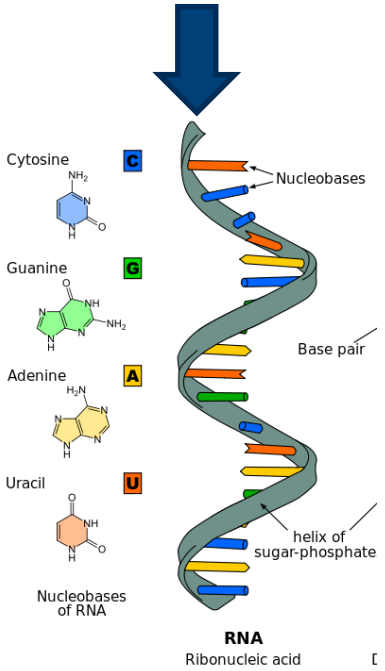

Key to successful real time RT-PCR
Sensitivity and specificity

Tissue sampling and RNA extraction

- **Choosing the right tissue and optimal extraction method for highest yield of RNA**

→**PMCV**

- RNA virus
- Detect both viral genome and viral transcripts
- Heart tissue standard, but also ovarian fluid, milt, kidney, spleen and gills can be used



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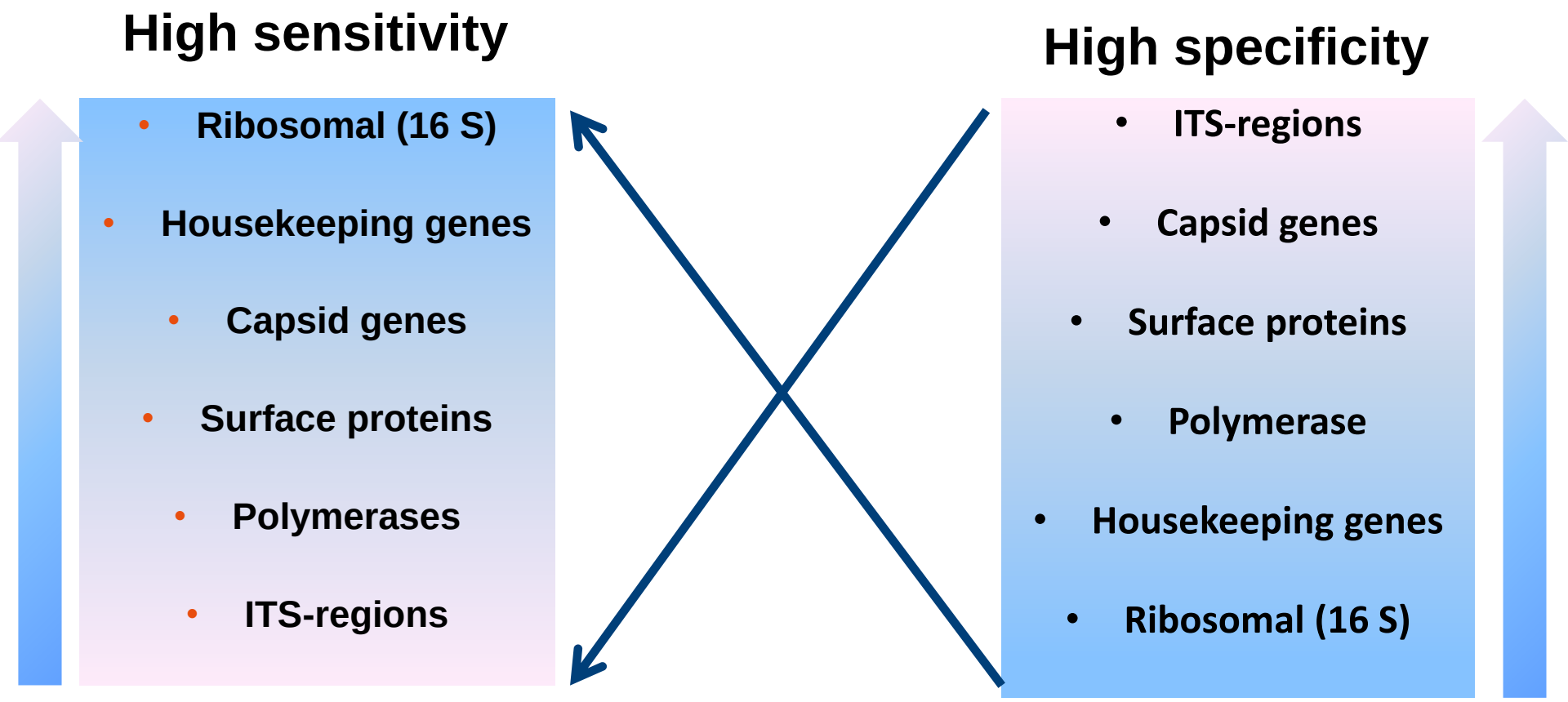
Assay design: Choose your target wisely



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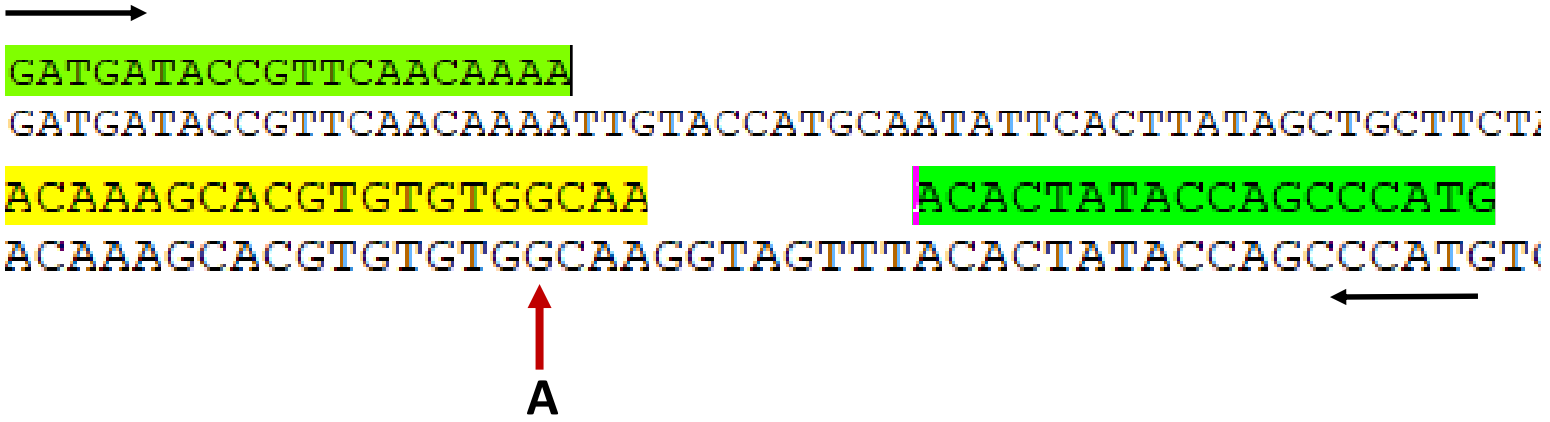
Assay design:



- Specific primers- and probes

→ **Optimize sensitivity and specificity**

→ **Do you have the right controls? Positive, negative, blank**

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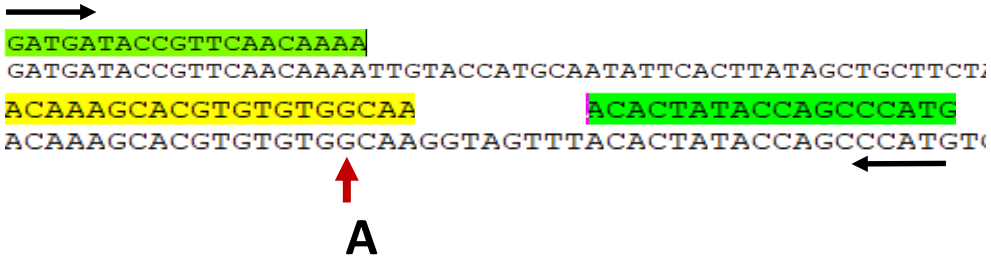
What affects sensitivity and specificity?

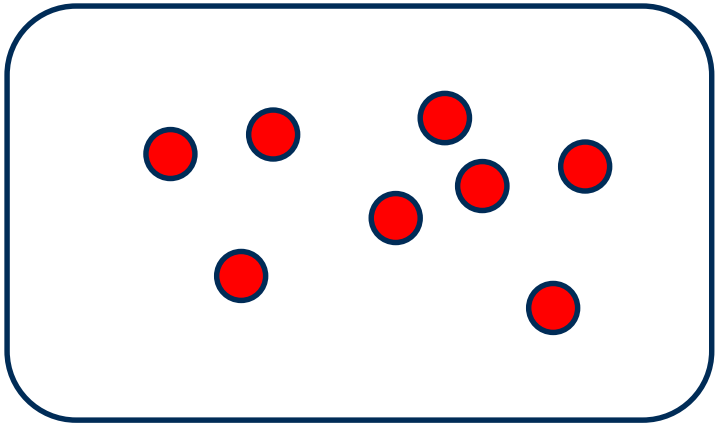
- **Size matters**
- Primers- and probes
- Target area

→ Optimizing reaction kinetics

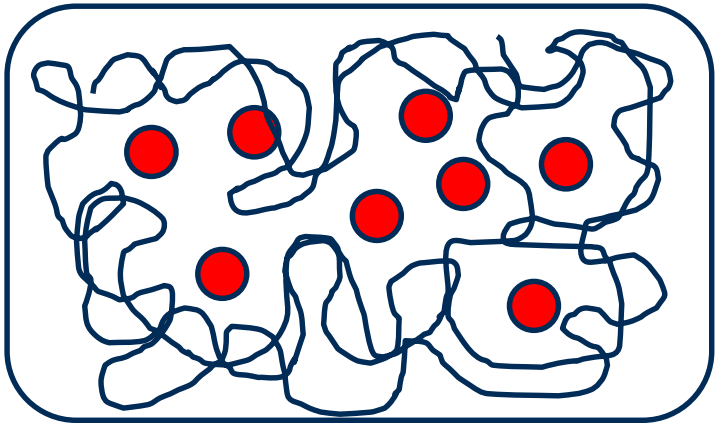


What is Cut-off, and why do we use it?

- **Analytical sensitivity**
- How much of the target can we detect without the presence of biological material



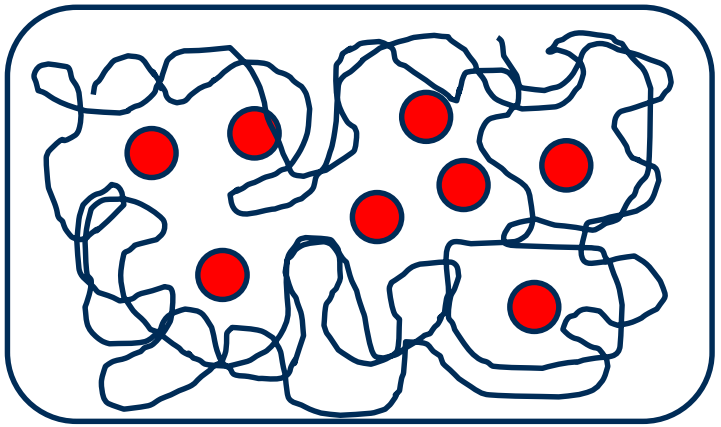
- **Diagnostic sensitivity**
- How much of the target can we detect in the presence of biological material (RNA from fish)



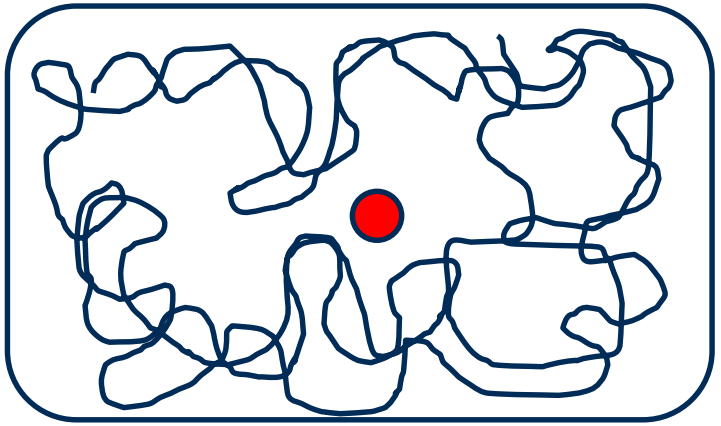
➡ Avoid false positive and false negative results

Cut-off and PMCV

- **Sea Phase**



- **Broodfish**
- **Juveniles**
- **Smolt**



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What is Cut-off, and why do we use it?

➢ Calculating limit of detection and Cut-off

				Analytical sensitivity Dilution: water		Diagnostic sensitivity Dilution: matrix	
	Dilution	Nr. copies pr. ul	Nr. copies reaction	Nr. positive	Ct-value (avg, n=10)	Nr. Positive	**Ct-value (avg, n=10)**
Start	1,00E-09	684,59					
1	2,00E-10	137,12	342,79	10	32,51	10	**32,43**
2	1,00E-10	68,56	171,40	10	33,57	10	**33,30**
3	5,00E-11	34,28	85,70	10	34,59	10	35,21
4	2,50E-11	17,14	42,85	9	35,76	8	**35,31**
5	1,25E-11	8,57	21,42	7	35,59	6	**36,11**
6	**6,25E-12**	**4,28**	**10,71**	**7**	**35,46**	**5**	**36,12**

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What is Cut-off, and why do we use it?

➢ **Limit of detection (LOD)**

- The lowest theoretical amount of template it is possible to detect

➢ **Cut- off**

- The lowest amount of template it is possible to detect and reproduce

→**Values above Cut-off does not necessarily mean that samples are negative**

Take home message:

- Real time RT-PCR: only pathogen detection
- Real time RT-PCR + Histology = Diagnosis
- Different labs = different methodology

Thank you for your attention!

- What affects realtime PCR?
    - Ekstraction method- RNA difficult
    - Make specific assays
    - Conserved to agent of interest
    - Size of primers and probe and the sequence area of detection?

- Difference between detection and diagnostics
- PCR is just detection
- Need tissue changes to determine/set a diagnosis

How do you document that a fish individual/group is negative?