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Experiments

Study number	Name/Description	Date
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SN0002	Control #2 w/7 primer sets	02.09.2020
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SN0004	Old vs new homemade buffer	03.09.2020
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SN0006	One-step PCR w/1X and 2X template conc.	14.09.2020
SN0007	One-step PCR w/Sarbeco and diff. Taq times	17.09.2020
SN0008	One-step RT-PCR of all primers	17.09.2020
SN0009	Finding optimal temp. for all primer sets	21.09.2020
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SN0013	Reducing RT and anneal/extension time	01.10.2020
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SN0028	New conc. of #21 and MS2, 2X template, gradient and 20 vs 25 µL	03.11.2020
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SN0029.2	SN0029.1 repeat with 5X RNA and 25 µL samples	03.11.2020
SN0030	Weekly run: setup30, all Covid-19 primer sets and MS2 IC	Week 46-
SN0031	Setup30 with 1X Trondheim RNA and 3X Spytt RNA	11.11.2020
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SN0033	Patients positive for Sars-Cov2 in Oslo RNA plate	12.11.2020
SN0034	Testing Bhadra et al (2020) protocol for RT with Taq only	19.11.2020
SN0035	Finding the optimal Mg^+ concentration with our mix	25.11.2020
SN0036	Finding the opt. Mg^+ concentration with our one-step and two-step mix	
SN0037	Testing the new and old batch of MS2 and TM primer/probe sets	02.12.2020
SN0038	Testing different concentration of Mg^+ with KrasX2 primer/probe set	02.12.2020
SN0039	Trying MS2 internal control in Oslo RNA and Trondheim RNA	02.12.2020
SN0040		
SN0041		
SN0042		

Trondheim #1 w/7 primer sets

SN0001 02.09.2020

Aim:

Run 1st RT-PCR with Trondheim #1 sample (cDNA) and 7 primer sets. All parameters are based on preliminary data from Per O. Ekstrøm.

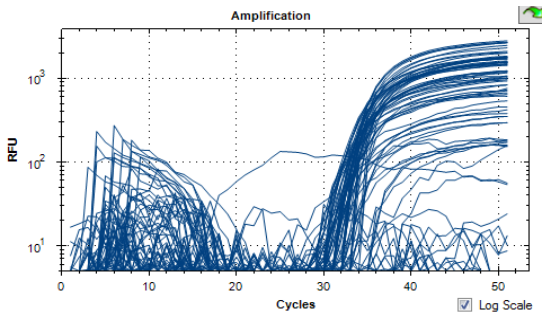
Mix:

Fragment: Sars CoV	**PCR**		Grad 50-63C	Date	02.09.2020	
PCR volum, µl	20		# of reactions		9	
	Working					
	solutions		Total volume 180 µl		Desired	
	concentration		Volum		concentration	
H_2O			155,16			
10X Thermopol uten MgSO4	0	mM MgCl	18,00			
$MgSO_4$	200	mM	1,80		2	mM
Primer forward	100	µM	0,72	Used 2,2 µL of each	0,4	µM
Primer reverse	100	µM	0,72		0,4	µM
Probe	100	µM	0,72		0,4	µM
dNTP	100	mM	0,72		400	µM
cDNA	10	ng			0,5	ng/µl
BSA	100	%	1,80		1	%
Dave Taq native	37,5	U/µl	0,36		0,075	U/µl
Mashup	7,5	U/µl			0,075	U/µl

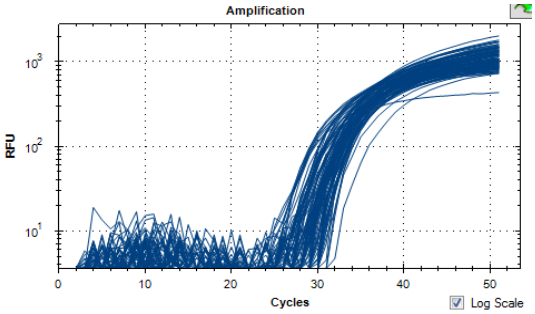
Fragment: Sars CoV 2	**PCR**			Date	02.09.2020	
PCR volum, µl	20		# of reactions		70	
	Working					
	solutions		Total volume 1400 µl		Desired	
	concentration		Volum		concentration	
H_2O			1083,60			
10X Thermopol #1	0	mM MgCl	140,00			
$MgSO_4$	200	mM	14,00		2	mM
Primer forward	100	µM			0,4	µM
Primer reverse	100	µM			0,4	µM
Probe	100	µM			0,4	µM
dNTP	100	mM	5,60		400	µM
cDNA	7	ng	140,00		0,7	ng/µl
BSA	100	%	14,00		1	%
Taq Hot	37,5	U/µl	2,80		0,075	U/µl
Mashup	7,5	U/µl	0,00		0	U/µl

Results:

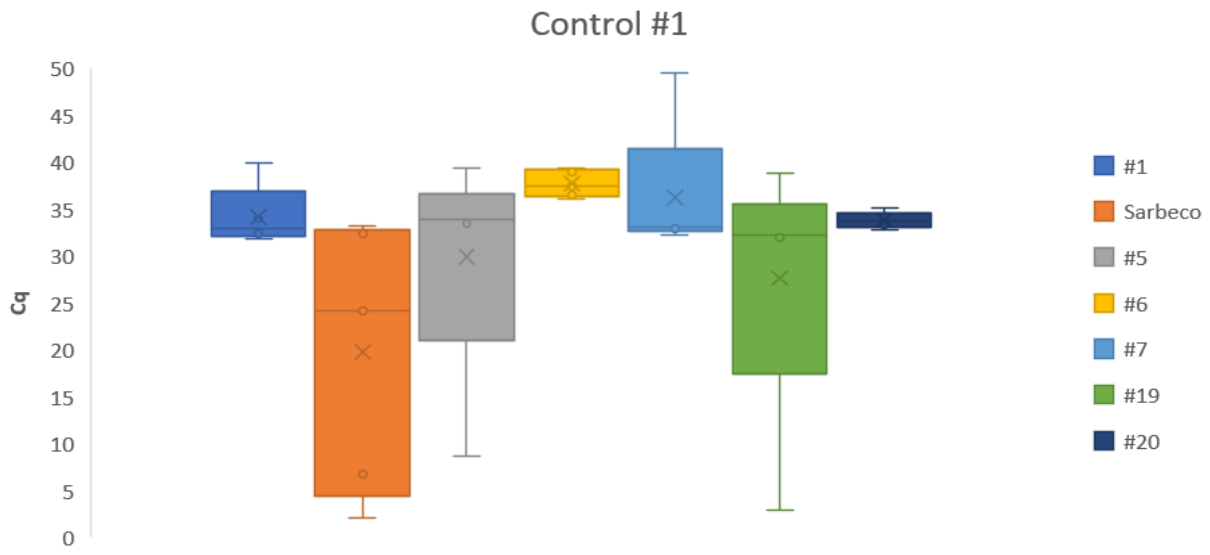
A



B



C



First RT-PCR. (A) A lot of background noise can be seen in the image. Reasons for this could be bubbles (remember to spin down plate at 1000 rpm before RT-PCR) or pipetting errors. **(B)** 2nd run with less noise. **(C)** Box plot of control sample results from the RT-PCR.

Conclusion:

Primer set #7 and #20 shows the most promising results, with low cycle values (Cq/Ct) and high fluorescence. Primer set #20 also seem more specific than most of the other sets.

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Control #2 w/7 primer sets

SN0002 02.09.2020

Aim:

Check if PCR results improves with 10 µL mix compared to 20 µL.
Used pre-made cDNA.

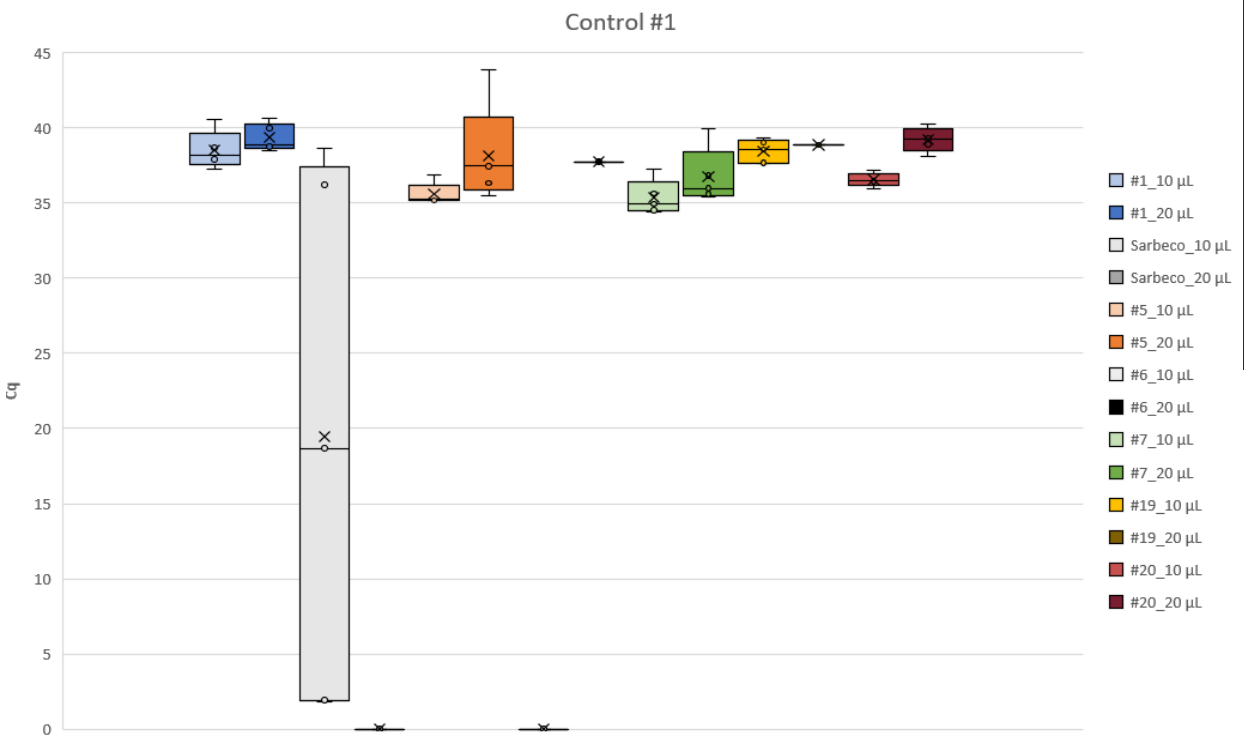
Mix:

Same as in SN0001, with some adjustments in regards to number of samples.

PCR protocol:

- 50-61°C temperature gradient
- 50 cycles

Results:



Conclusion:

Some values were not included in the box plot as they represented outliers (a lot of noise).

Homemade vs kit buffer

SN0003 03.09.2020

Aim:

See if homemade buffer is as good/better than a kit buffer.
Used pre-made cDNA from Control #1 RNA and Trondheim RNA.

Mix:

PCR run with Per, 03.09.2020. Tested 2 different volumes (1-2 µL) of cDNA (after synthesis, template volume pipetted directly into wells) and homemade buffer vs. kit buffer. Samples were tested with primer #7 and #20, as previous experiments have shown that these primers gives best effect (steepest curves, little background noise).

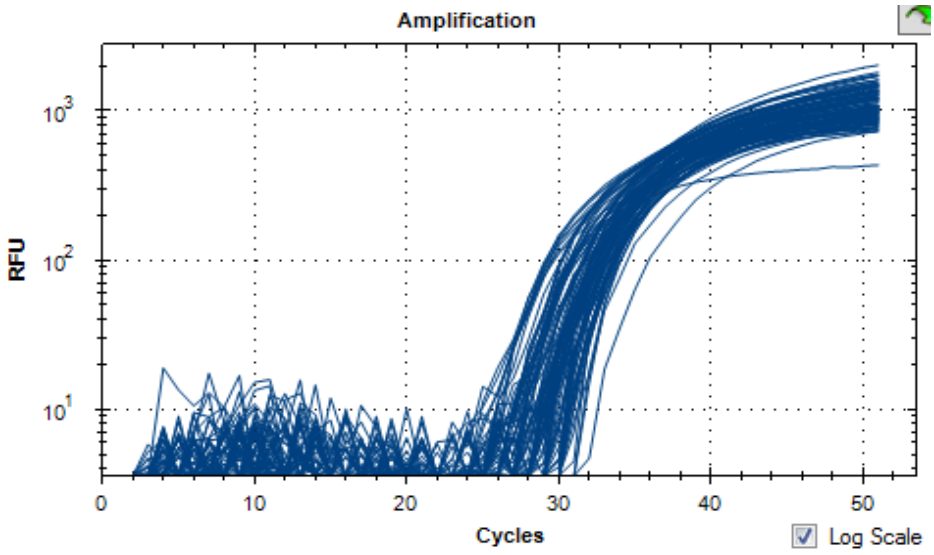
Fragment: Sars CoV 2 #7/20	**PCR**			Date	03.09.2020	
PCR volum, µl	20		# of reactions		50	
	Working					
	solutions		Total volume 1000 µl		Desired	
	concentration		Volum		concentration	
H2O			862.00			
10X Thermopol #1	0	mM MgCl	100.00			
MgSO4	200	mM	10.00		2	mM
Primer forward	100	µM	4.00		0.4	µM
Primer reverse	100	µM	4.00		0.4	µM
Probe	100	µM	4.00		0.4	µM
dNTP	100	mM	4.00		400	µM
cDNA	10	ng	0.00	Adjust to 1X or 2X	0	ng/µl
BSA	100	%	10.00		1	%
Dave Taq native	37.5	U/µl	2.00		0.075	U/µl
Mashup	7.5	U/µl	0.00		0	U/µl

PCR protocol:

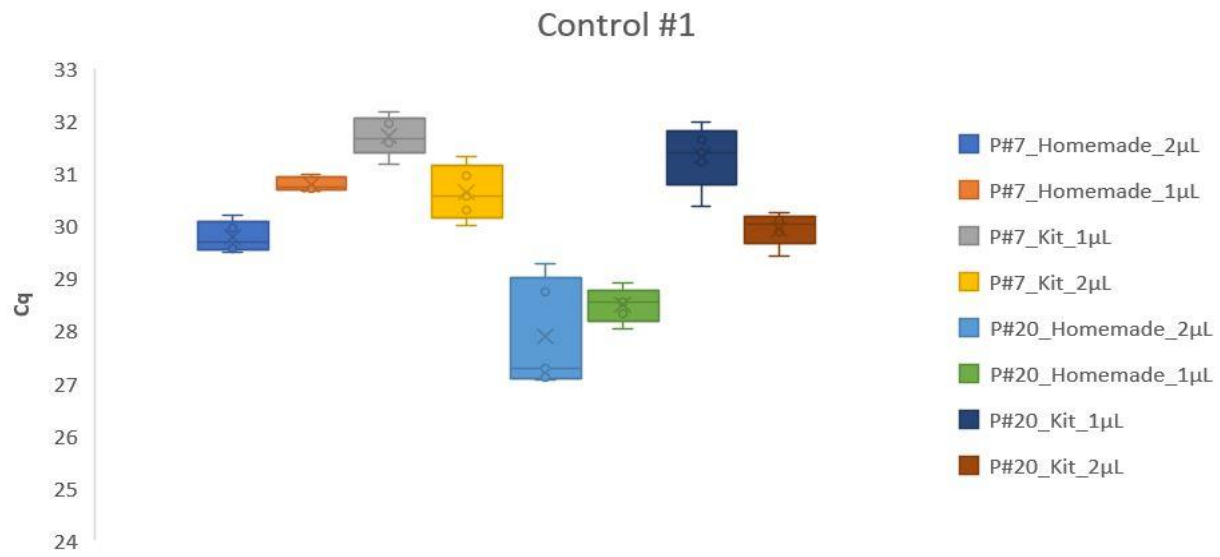
- Same PCR settings as in SN0002

Results:

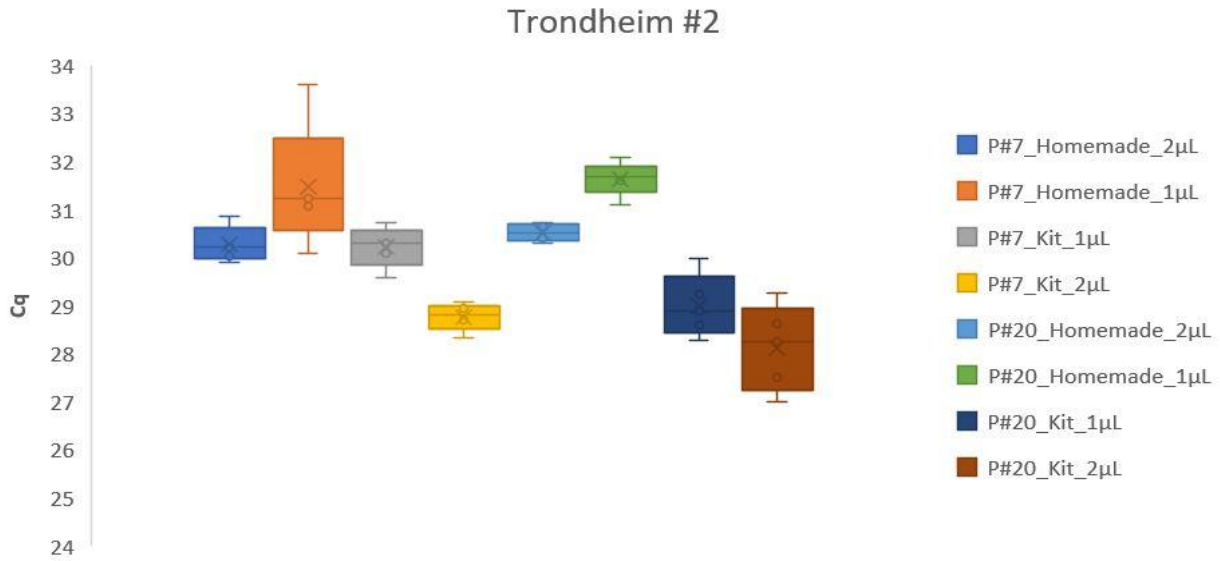
A



B



C



Homemade vs kit buffer. (A) Regression-fitted amplification curve from experiment. **(B)** Box plot of Cq values when different amounts of cDNA from Control #1 RNA was used with primer set #7 or #20, and with different types of buffers. **(C)** Same conditions as in (B), except for the template. Here, cDNA from "Trondheim RNA" was used (purified RNA, received from Dr. Magnar Bjørås).

Conclusion:

- Homemade buffer gave better restults in Control cDNA samples than kit buffer, but not in Trondheim samples.
- Overall, 2X template gave more specific values with lower Cq - not that surprising as more viral RNA gets detected earlier/better.
- We conclude that we can safely use the homemade 10X Thermopol buffer (with Mg^{+}) for later experiments.
- I added $MgSO_4$ by mistake in all buffers, but it didn't seem to induce any notable differences.

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Old vs new homemade buffer

SN0004 03.09.2020

Aim:

Per made a really good batch of homemade buffer, but it is almost empty - we want to check if the fresh one is just as good for usage (the pH meter was broken when the latter was made).

Mix:

Used primer sets #7 or #20, in addition to 2 different template concentrations (1X and 2X).

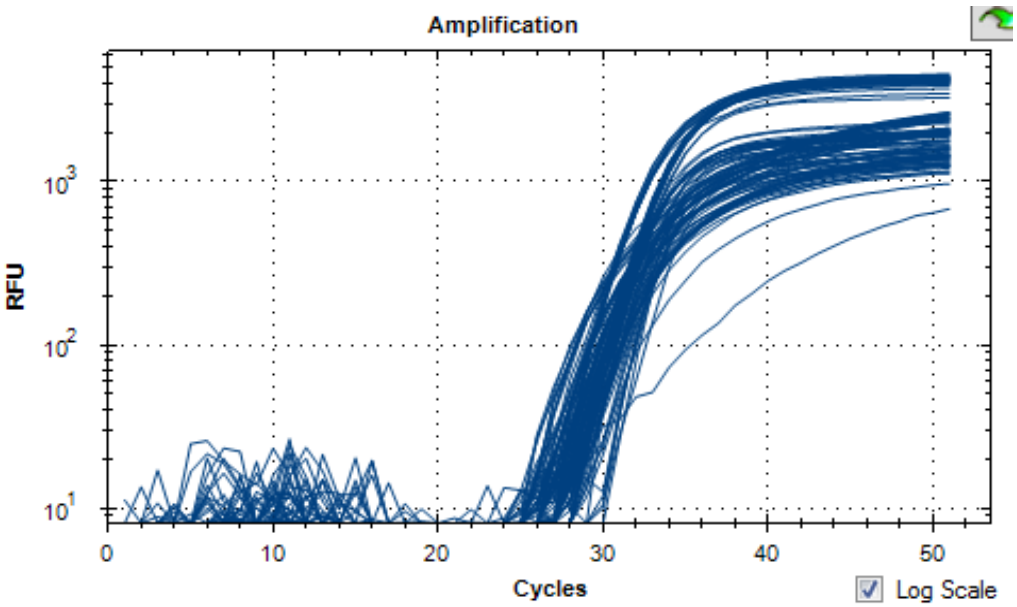
Fragment: Sars CoV 2 #7/20	**PCR**			Date	03.09.2020	
PCR volum, µl	20		# of reactions		25	
	Working					
	solutions		Total volume 500 µl		Desired	
	concentration		Volum		concentration	
H2O			431.00	or 436		
10X Thermopol old or new	0	mM MgCl	50.00	Only 1 buffer w/Mg^+		
MgSO4	200	mM	5.00	or 0	2	mM
Primer forward	100	µM	2.00		0.4	µM
Primer reverse	100	µM	2.00		0.4	µM
Probe	100	µM	2.00		0.4	µM
dNTP	100	mM	2.00		400	µM
cDNA	10	ng	0.00	Adjust to 1X or 2X	0	ng/µl
BSA	100	%	5.00		1	%
Dave Taq native	37.5	U/µl	1.00		0.075	U/µl
Mashup	7.5	U/µl	0.00		0	U/µl

PCR protocol:

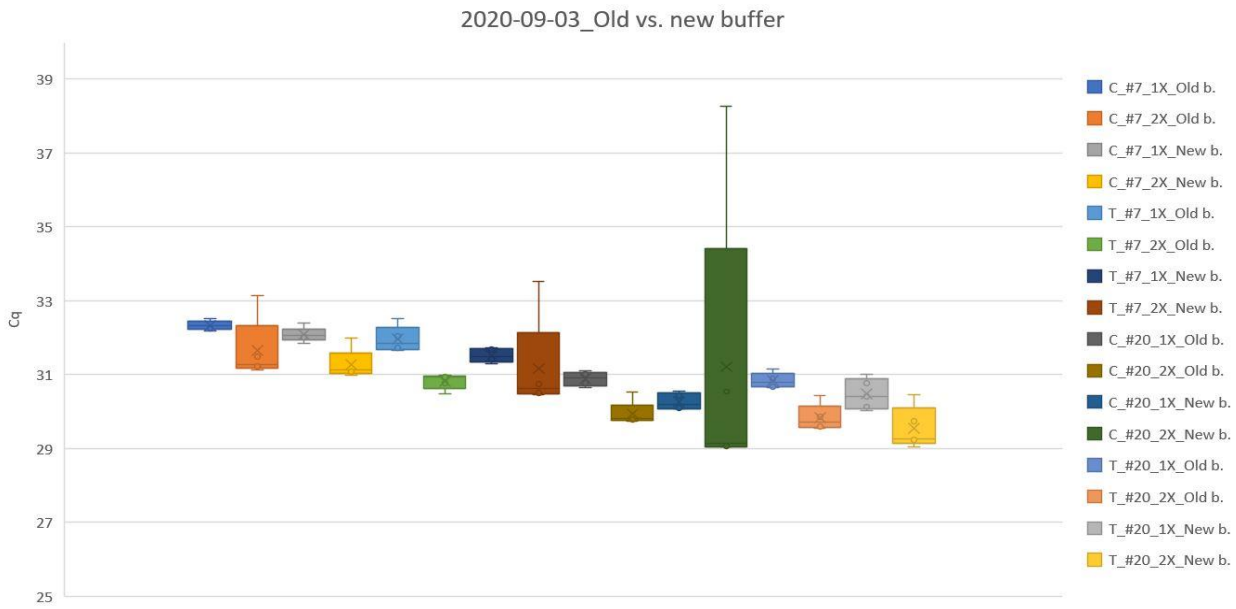
- Same PCR settings as in SN0002

Results:

A



B



Old vs new homemade buffer. (A) Regression-fitted amplification curve from experiment. **(B)** Box plot from the experiment.

Conclusion:

- The results were quite varying but it seems that the fresh buffer gives just as good results as the old (good) batch. We can continue with the new one when the old runs out.
- 2X template gives a bit better Cq, consistent with out previous findings (see SN0003).

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One-step PCR, test of Taq Native vs Platinum

SN0005 11.09.2020

Aim:

To test the possibility of doing the whole reaction in one step.

Mix:

Same as in SN0001, with some adjustments:

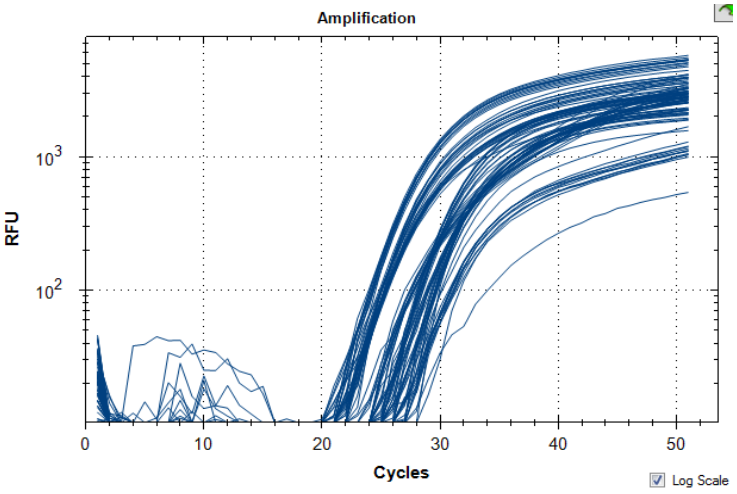
- RNA (w/MashUp RT) vs cDNA (w/o RT)
  1-step 2-step
- Native Taq (Nat) vs Platinum Taq (Plat)
- Primer set #7 vs #20
- 8 conditions in total, 12 samples per condition

PCR protocol:

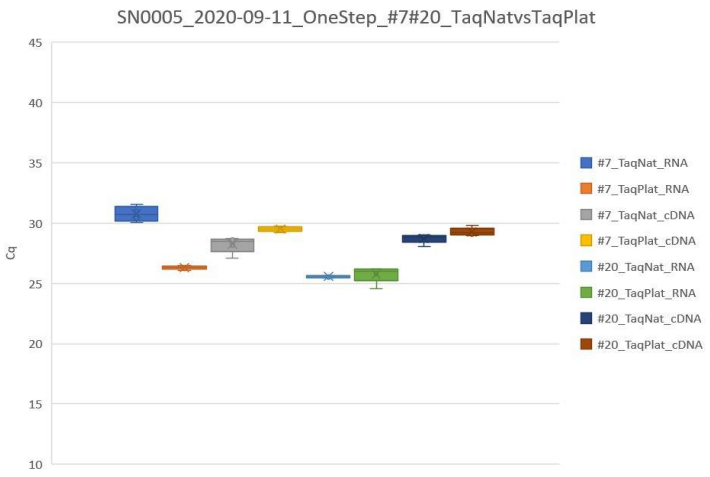
- Same PCR settings as in SN0002
- 10 min cDNA synthesis at the start of the PCR protocol

Results:

A



B



One-step PCR test with different Taq polymerases. (A) Regression-fitted amplification curve from the experiment. **(B)** Box plot from the experiment.

Conclusion:

- No large difference between native and platinum Taq in #20 samples.
- Native Taq shows up a bit later in #7 samples but in parallel with Platinum Taq.

  This should not be affected by the temperature gradient as the differences are too small.
- From these results, it looks like one-step RT-PCR with our components work!

  Should be repeated for verification.

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One-step PCR w/1X and 2X template conc.

SN0006 14.09.2020

Aim:

Repeat SN0005 to verify that one-step RT-PCR works, and check whether 2X template concentration improves the results.

Mix:

Same as in SN0005, with some adjustments:

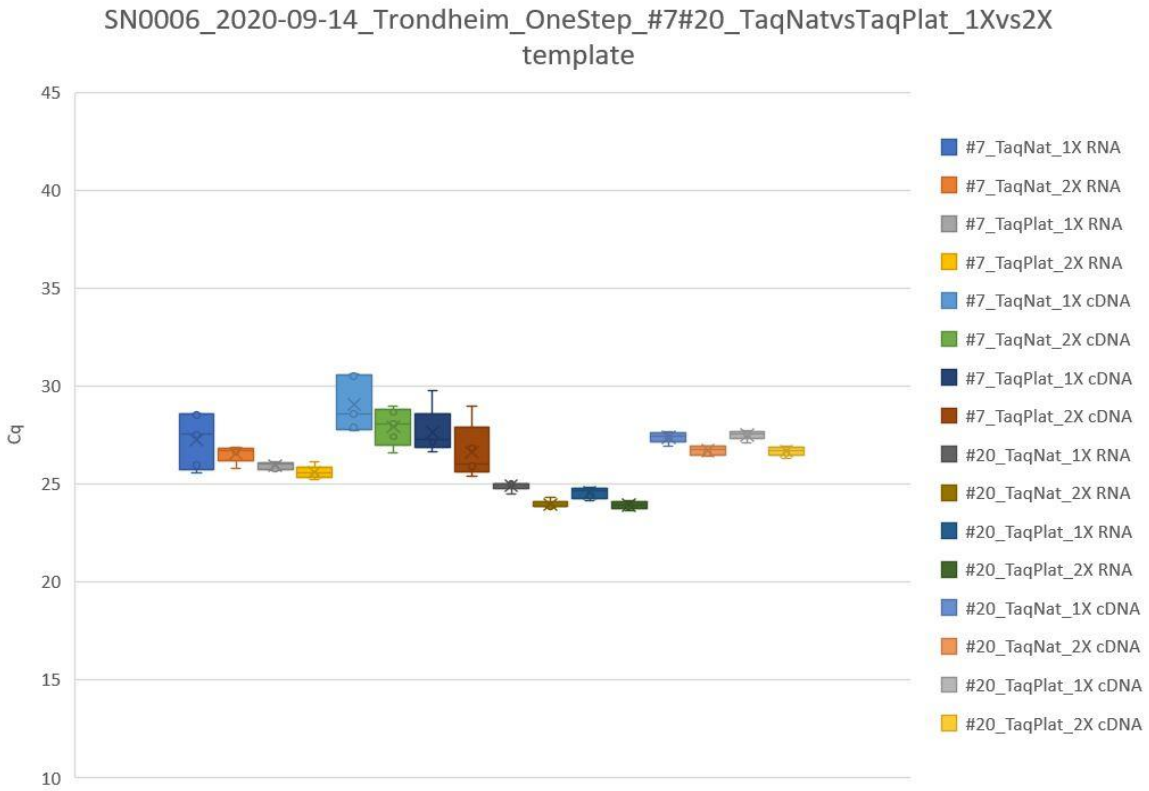
- Either 1X or 2X template concentration

PCR protocol:

- Same as in SN0005

Results:

A



Conclusion:

- Could try to reduce cDNA synthesis time from 10 min to 5 min.
- Did not have enough Plat Taq/Hot start - only RNA samples got this polymerase, all cDNA samples got Nat Taq (some Plat Taq got diluted (2 µL + 1.9 µL H_2O)).
- In general, 2X works better than 1X template
- The box plots do not show any significant difference between Nat and Plat Taq
- RNA samples have lower Cq/Ct than cDNA samples (more copies)
- Primer #20 shows most specificity/least variance
- Row 1 had no template (negative control). In well H01 (grey box in box plot) some fluorescence was detected at ~35 Cq, which have been excluded in the figure.

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One-step PCR w/Sarbeco and diff. Taq times

SN0007 17.09.2020

Aim:

As one-step RT-PCR worked so far, we want to test the settings/mix with Sarbeco, the primer set used in standard testings in Norway. In this experiment, we also try to reduce the cDNA synthesis time to 5 min and include both 5 and 10 min incubation in the experiment (sealing half of the plate).

Mix:

Fragment: Sars CoV Sarb	**PCR**		Grad 50-63C	Date	17.09.2020	
PCR volum, µl	20		# of reactions		50	
	Working					
	solutions		Total volume 1000 µl		Desired	
	concentration		Volum		concentration	
H_2O			802.00			
10X Thermopol uten $MgSO_4$	0	mM MgCl	100.00			
$MgSO_4$	200	mM	10.00		2	mM
Primer forward	100	µM	4.00		0.4	µM
Primer reverse	100	µM	4.00		0.4	µM
Probe	100	µM	4.00		0.4	µM
dNTP	100	mM	4.00		400	µM
RNA/cDNA	10	ng	50.00		0.5	ng/µl
BSA	100	%	10.00		1	%
Dave Taq native	37.5	U/µl	2.00		0.075	U/µl
Mashup	7.5	U/µl	10.00		0.075	U/µl

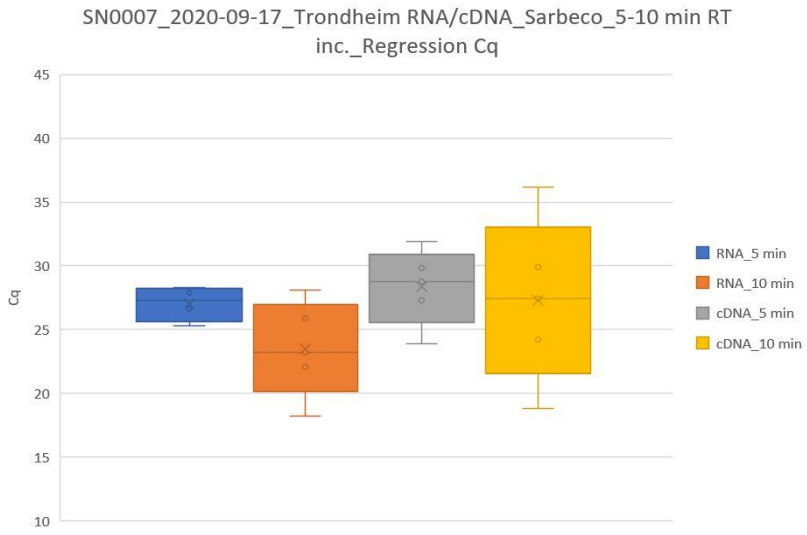
S.O.E.: Samples with 10 min RT incuation time got twice as much Taq! Repeated experiment.

PCR protocol:

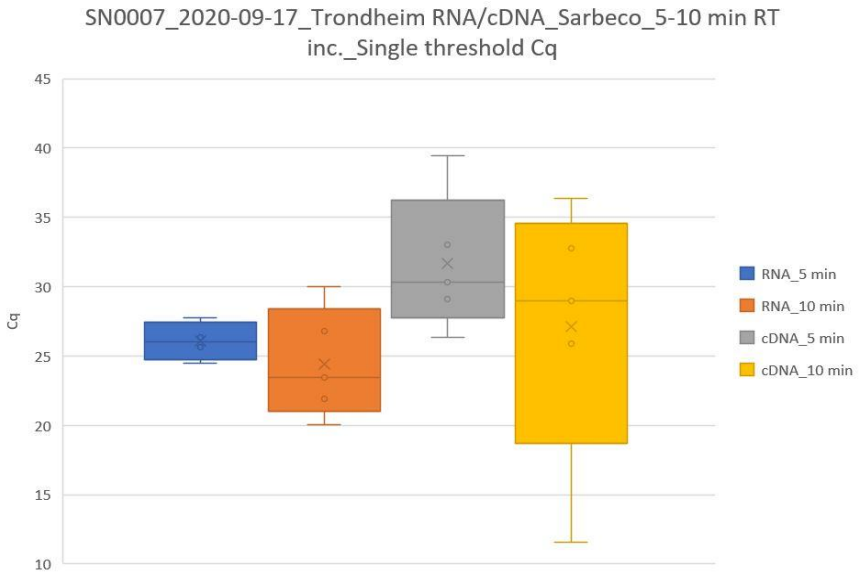
- Same as in SN0005, except that 10 min samples were incubated at 42°C for 5 min prior to adding the 5 min samples (no enzyme loaded in 5 min samples at the start).
  Plate was partly sealed with tape (Clas Ohlson).

Results:

A



B



One-step RT-PCR with Sarbeco primer set and differen Taq polymerase times. (A) Box plot based on regression-fitted values. **(B)** Box plot from based on single threshold Cq values.

Conclusion:

- Although the 10 min incubation samples have lower Cq values, the 5 min samples are more specific.
- We will use 5 min RT incubation time from now on.

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One-step RT-PCR of all primers

SN0008 17.09.2020

Aim:

Test all primers with the latest set-up (including 5 min RT incubation)
Used pre-mixed primer/probe mix from strip.

Mix:

I loaded the primer/probe mix into wells before adding the mastermix.

One-step RT-PCR

Sample		Date	17.09.2021
PCR volum, µL	20	**# of reactions**	100

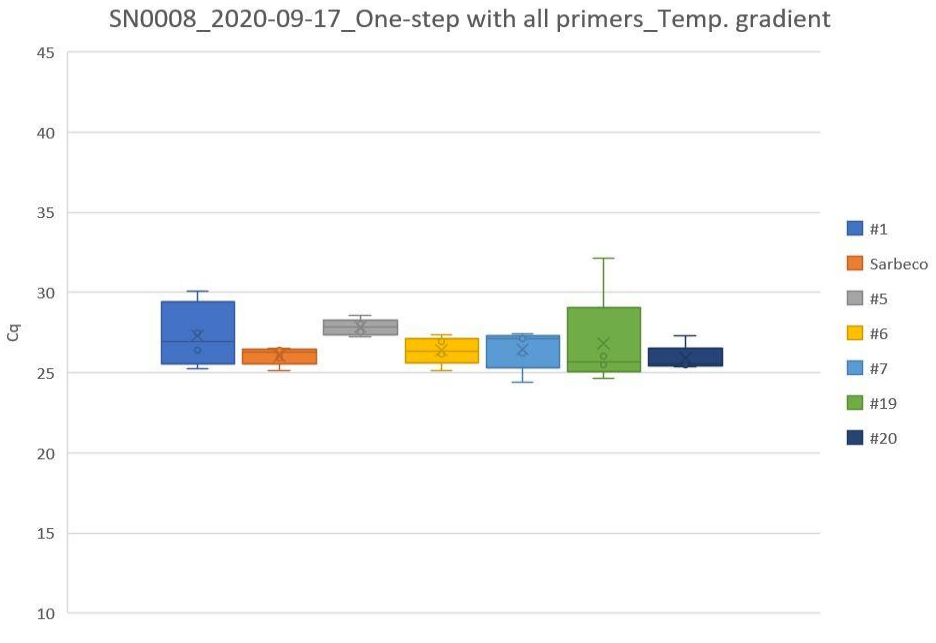
Reagents	Working sol. conc.		Total volume 2000 µL Volume		Desired conc.	
H_2O			1627.98			
10X Thermopol uten $MgSO_4$	0		200.00			
$MgSO_4$	200	mM	20.00		2	mM
Primer forward	100	µM	0.01	Used pre-mixed primer/probe mix	0.0004	µM
Primer reverse	100	µM	0.01		0.0004	µM
SYBR green	100	µM	0.01		0.0004	µM
dNTP	100	mM	8.00		400	µM
RNA	10	ng	100.00		0.5	ng/µl
BSA	100	%	20.00		1	%
Dave Taq native	37.5	U/µl	4.00		0.075	U/µl
Mashup	7.5	U/µl	20.00		0.075	U/µl

PCR protocol:

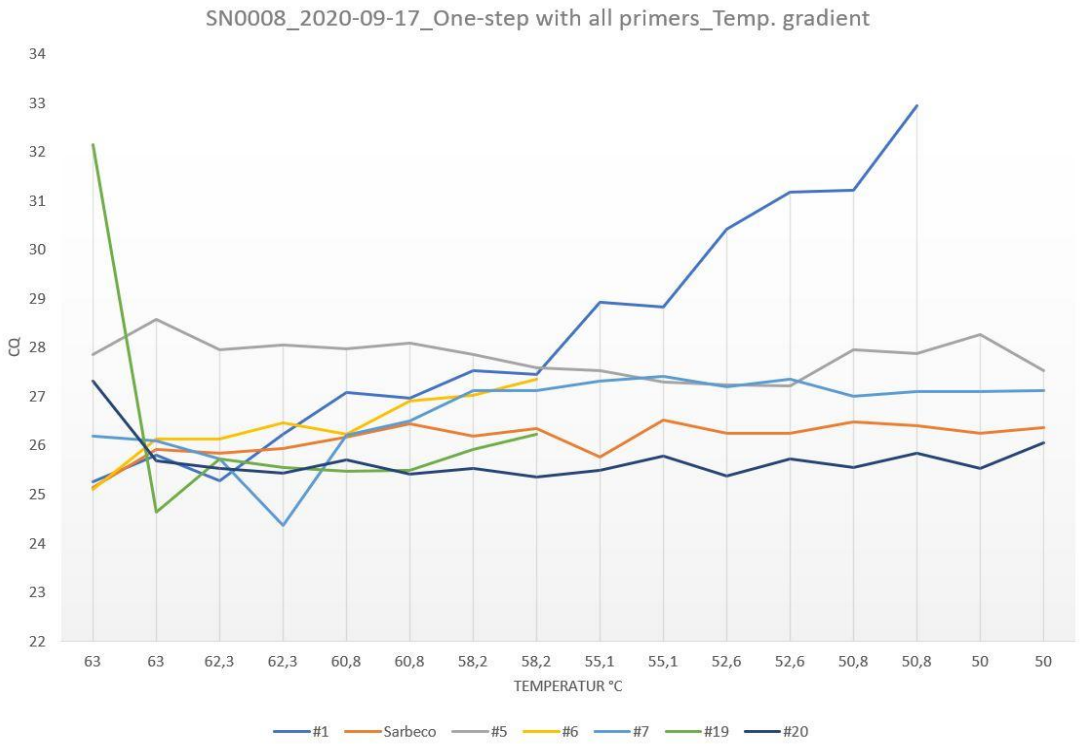
- Same as in SN0006, with only 5 min RT incubation time

Results:

A



B



One-step RT-PCR of all primers. (A) Box plot based on regression-fitted values. **(B)** The graph shows optimal temperature for annealing during RT-PCR for each primer/probe set.

Conclusion:

- We will continue to use the temperature that gives the best (i.e. steepest) curve for each primer (see continuation in SN0009).

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Finding optimal temp. for all primer sets

SN0009 21.09.2020

Aim:

To compare all primer/probe sets with their resapective optimal conditions.
Optimal temperature are based on data from project SN0008.

Mix:

I used the same mix as in SN0008 but with some adjustments:

One-step RT-PCR

Fragment:	**Trondheim RNA**		Date	21.09.2020
PCR volum, µL	20	Temp. Gradient 55-62°C	**# of reactions**	12

Reagents	Working sol. conc.		Total volume 240 µL Volume		Desired conc.	
H_2O			192.48			
10X Thermopol uten $MgSO_4$	0		24.00			
$MgSO_4$	200	mM	2.40		2	mM
Primer forward	100	µM	0.96	Or 2.88 µL from mix	0.4	µM
Primer reverse	100	µM	0.96		0.4	µM
SYBR green	100	µM	0.96		0.4	µM
dNTP	100	mM	0.96		400	µM
RNA/cDNA	10	ng	12.00	Add after blank	0.5	ng/µl
BSA	100	%	2.40		1	%
Dave Taq native	37.5	U/µl	0.48		0.075	U/µl
Mashup	7.5	U/µl	2.40		0.075	U/µl

PCR protocol:

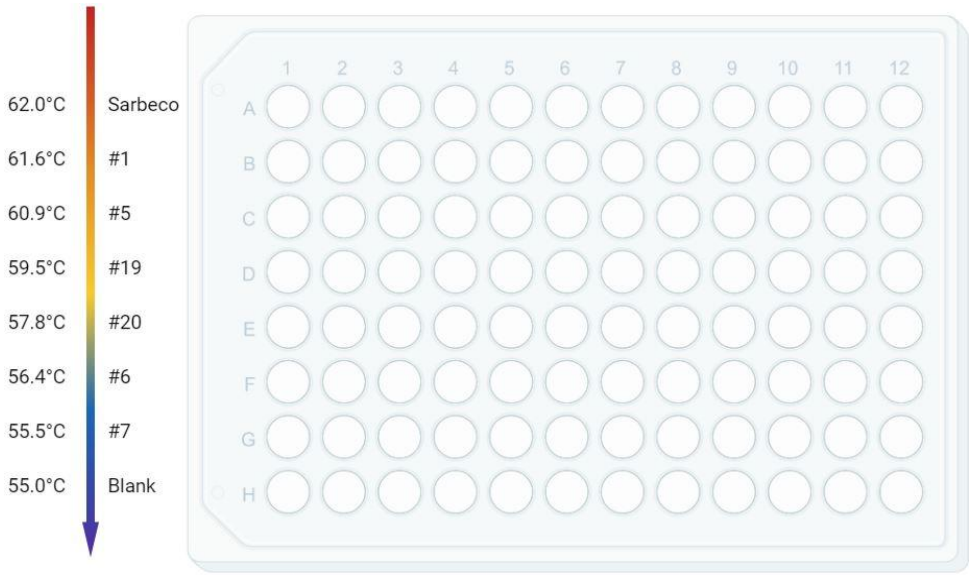
- Same as in SN0006, but with a temperature gradient fit for the different primer/probe sets:

Primer/probe set	Optimal temperature (defined by steepest curve)
# Sarbeco	62.3 - 63
# 1	60.8 - 63
# 5	55.1 - 63
# 6	55.1 - 58
# 7	curves looked good at all temperatures in gradient
# 19	52.6 - 62.3
# 20	52.6 - 58.2

From this, the following temperature gradient was created and included in the thermal (PCR) protocol:

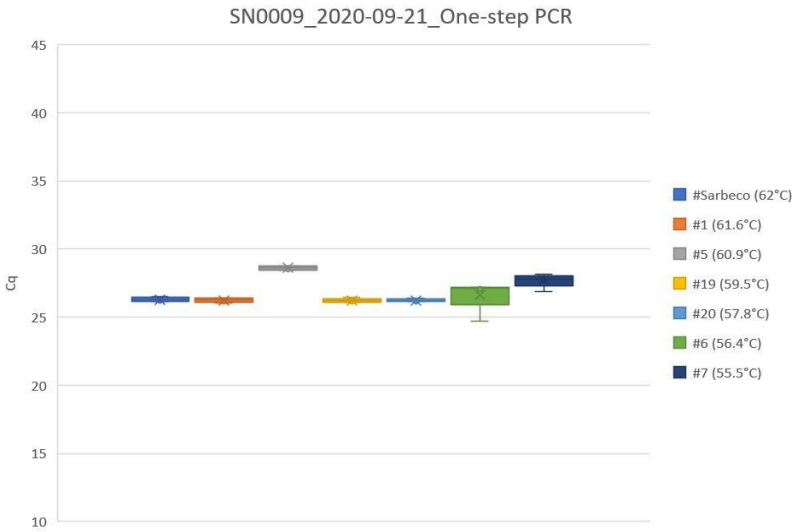
See next page.

Temperature gradient:

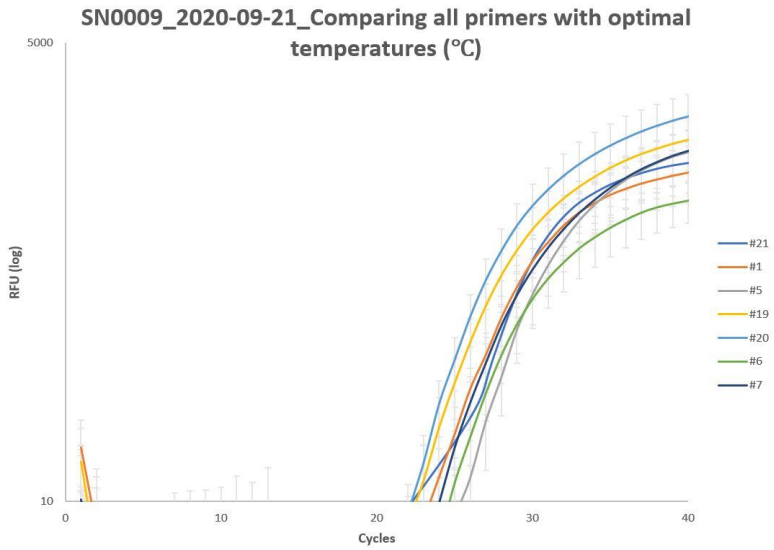


Results:

A



B



Finding the optimal temperature for all primer/probe sets. (A) Box plot from the experiment. **(B)** Graph showing the regression-fitted curves of all samples (standard deviation included).

Conclusion:

- We will keep testing the primer/probe sets as we did in this project by including the temperature gradient in the thermal (PCR) protocol.
- Optimal temperature did not necessarily match the melting temperature for all primer/probe sets (tested by manufacturer with NaCl).

Reducing extension time to 30 seconds

SN0010 21.09.2020

Aim:

Try to reduce extension from 1 minute to 30 seconds (s), to save PCR time.

Mix:

One-step RT-PCR

Fragment:	Control #1		Date	21.09.2020
PCR volum, µL	20	Temp. Gradient 55-62°C	# of reactions	13

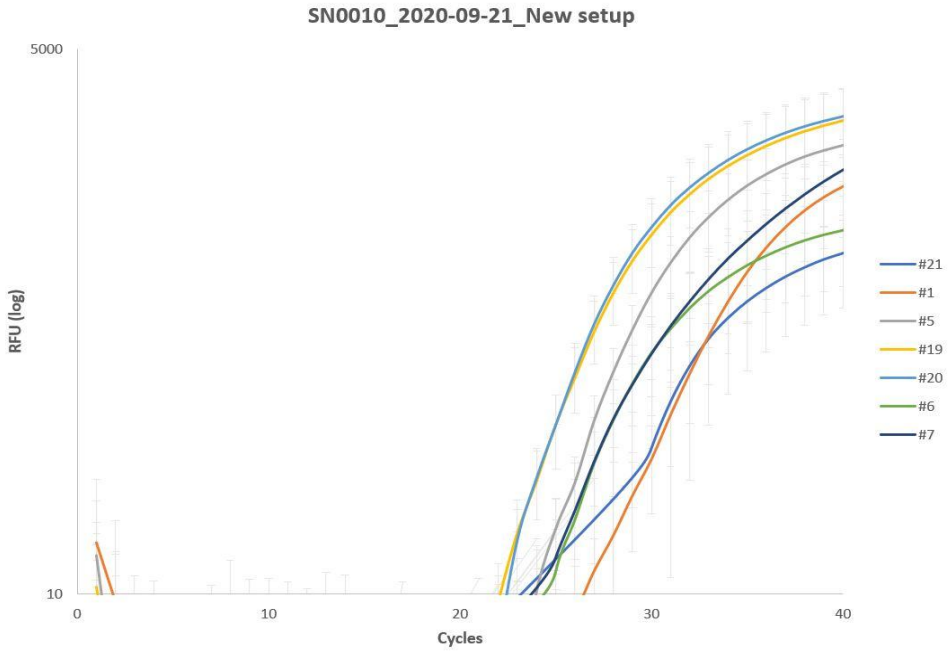
Reagents	Working sol. conc.		Total volume 260 µL Volume		Desired conc.	
H_2O			208.52			
10X Thermopol uten $MgSO_4$	0		26.00			
$MgSO_4$	200	mM	2.60		2	mM
Primer forward	100	µM	1.04	Or 3.12 µL from mix	0.4	µM
Primer reverse	100	µM	1.04		0.4	µM
SYBR green	100	µM	1.04		0.4	µM
dNTP	100	mM	1.04		400	µM
RNA/cDNA	10	ng	13.00	12.00	0.5	ng/µl
BSA	100	%	2.60		1	%
Dave Taq native	37.5	U/µl	0.52		0.075	U/µl
Mashup	7.5	U/µl	2.60		0.075	U/µl

PCR protocol:

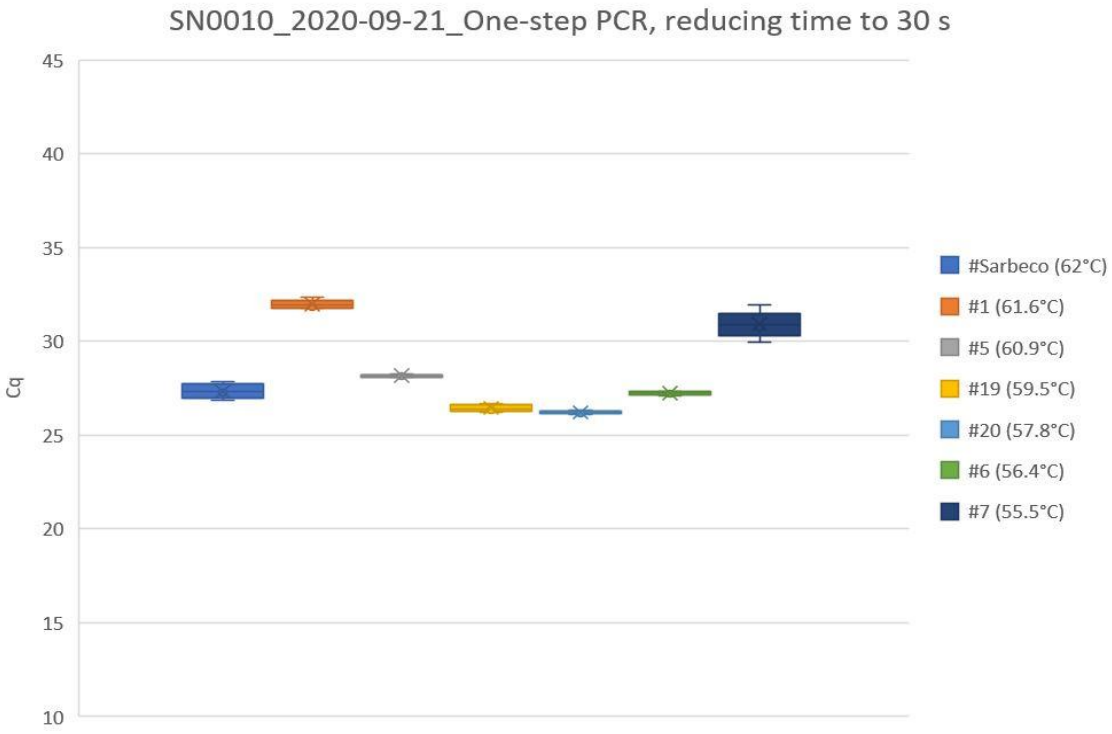
- Same as in SN0009

Results:

A



B



Reducing extension time to 30 seconds. (A) The graph shows the new set-up for all primer/probe sets, including the reduced extension time. **(B)** Box plot of the experiment.

Conclusion:

- The set-up optimalized so far will be called "Set-up 10" and includes:
  - Homemade kit
  - Temperature gradient when using different primer/probe sets
  - One-step RT-PCR thermal protocol with 5 min cDNA synthesis time and 30 seconds extension time