RPA_1 Initial Characterization

Project: Lab notebook
Authors: Marijn van den Brink
Created at: 2020-08-04T08:04:22.860614+00:00

MONDAY, 3/8/2020

Experimentalist(s): Tim and Sinisha.

RPA reactions were performed following protocol RPA_1. Saccharomyces cerevisiae genome was used instead of the pre-ordered dsDNA templates of the different targets.

Methods
Followed the RPA_1 Initial Confirmation protocol with the following primer combinations, all in duplicates:

Table1

	A	B	C	D	E
1	**Forward primer**	**Reverse primer**	**Template concentration**	**Product concentrations (ng/µL)**	**Product duplicate concentrations (ng/µL)**
2	SC2_F_p2_exp	SC2_R_Nt.AlwI_EAD 2+3A	2x dilution of 6.7 ng/µL	8.3	17.3
3	SC2_F_p2_exp	SC2_R_Nt.BsmAI_EAD 2+3A	2x dilution of 6.7 ng/µL	5.4	5.2
4	SC2_F_p2_exp	SC2_R_Nt.BspQI_EAD 2+3A	2x dilution of 6.7 ng/µL	5.9	38.8
5	SC2_F_p2_exp	SC2_R_Nt.BstNBI_EAD 2+3A	2x dilution of 6.7 ng/µL	33.4	23.4

Table2

	A	B
1	**Positive control concentration (ng/µL)**	**Negative control concentration (ng/µL)**
2	8.2	14.4

A 2% agarose gel was run for 40 min. with 12µL of a solution containing 24µL of sample and 6µL of Loading buffer for each sample.

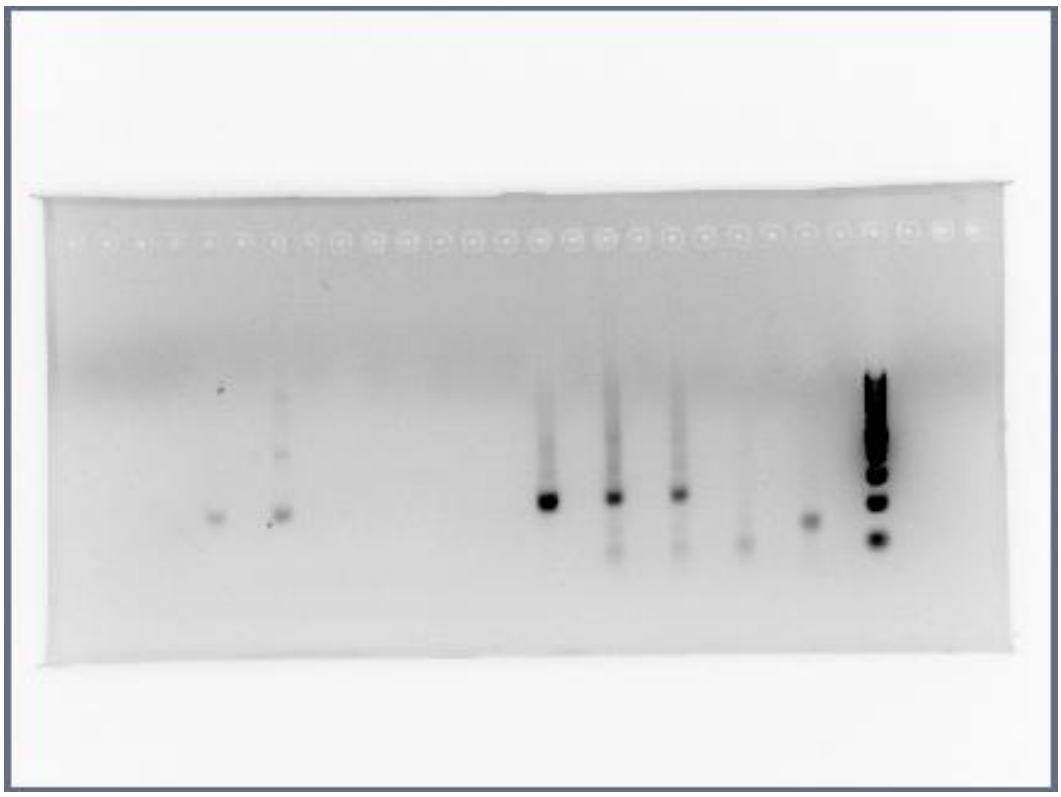
Nanodrop Results
See tables above for samples and controls.

Gel Electrophoresis Results
As the negative (lane 21) and positive (lane 23) control still showed a vague band and many of the primers sets showed no bands, we decided to redo the experiments on 4-8-2020. As the last primer combination showed promising results both on the gel and Nanodrop, we will use this again.

- Lane 5 and 7: SC2_F_p2_exp and SC2_R_Nt.AlwI_EAD 2+3A primer pair duplicates both show a band at the expected height on the gel
- Lane 9 and 11: SC2_F_p2_exp and SC2_R_Nt.BsmAI_EAD 2+3A primer pair duplicates both show no bands. We therefore expect that this primer pair does not work

- Lane 13 and 15: SC2_F_p2_exp and SC2_R_Nt.BspQI_EAD 2+3A primer pair duplicates only show a clear band in lane 12. We expect that something went wrong during the preparation of one of the duplicates.
- Lane 17 and 19: SC2_F_p2_exp and SC2_R_Nt.BstNBI_EAD 2+3A primer pair duplicates both show a band at the expected height on the gel.

RPA_init Confirmation.jpg



RPA_1 Initial Confirmation (2)

Project: Lab notebook
Authors: Tim van den Akker
Created at: 2020-08-04T17:26:33.587302+00:00

TUESDAY, 4/8/2020

RPA reactions were prepared following protocol RPA_1.

Methods

The experiments of the previous day have been repeated with the following changes:

Table1

	A	B	C	D	E
1	**Forward primer**	**Reverse primer**	**Template concentration**	**Product concentrations (ng/µL)**	**Product duplicate concentrations (ng/µL)**
2	SC1_F_p1	SC1_R_Nt.AlwI_EAD 2+3A	5x dilution of 6.7 ng/µL	38.5	46.8
3	SC1_F_p1	SC1_R_Nt.BstNBI_EAD 2+3A	5x dilution of 6.7 ng/µL	45.8	7.8
4	SC2_F_p2_exp	SC2_R_Nt.AlwI_EAD 2+3A	5x dilution of 6.7 ng/µL	35.8	10.2
5	SC2_F_p2_exp	SC2_R_Nt.BstNBI_EAD 2+3A	5x dilution of 6.7 ng/µL	35.0	9.6

Table2

	A	B
1	**Positive control concentration (ng/µL)**	**Negative control concentration (ng/µL)**
2	53.8	48.5

- Template stock concentration 5x dilution instead of 2x dilution
- The MgOAc was added directly into the tube instead of the lid.
- For the last step in the PCR cleanup, RNAse/DNase Free water had been heated to 42 degrees before use.
- A 2% agarose gel was used and run for 40 minutes with 12µL from a solution contain 24µL of sample and 6µL of Loading buffer for each sample.

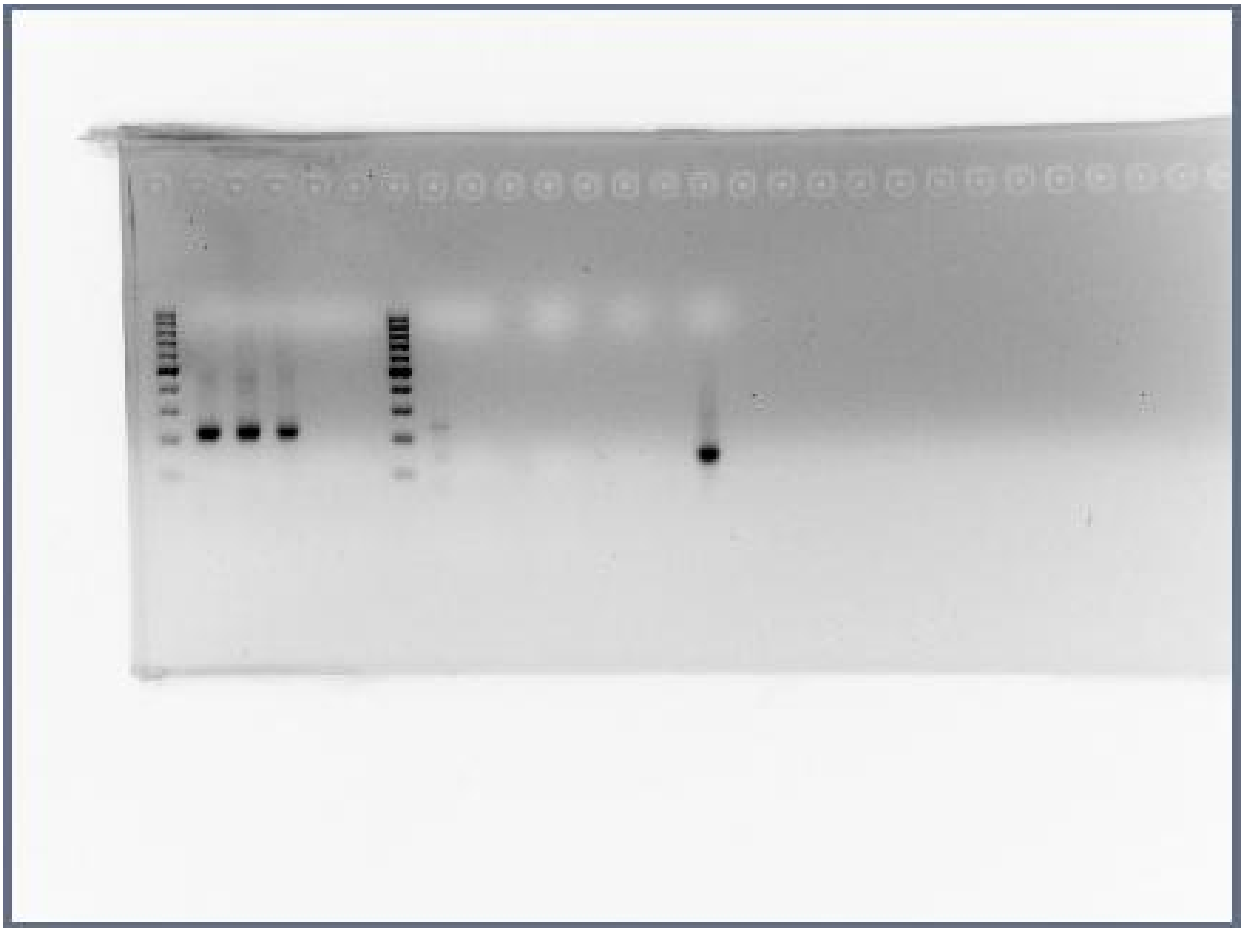
Nanodrop Results

See tables for samples and controls

Gel Electrophoresis Results

- The primer pairs in row 1 and 2 of Table 1 (lanes 8, 9, 11 and 13), which were also used on 3-8-2020 showed no bands.
- The primer pairs in row 3 of Table 1 (lanes 2 and 3), showed clear bands for both duplicates (these will also be used in RPA_2 Pyrophosphate detection)
- The primer pairs in row 4 of Table 1 (lanes 4 and 5), showed only a clear band for one the duplicates
- While the negative control (lane 6) showed a high concentration when measured with the Nanodrop, it did not show up in the gel.
- The positive control showed a clear band (lane 15).

2020-8-4 RPA initial confirmation day 2.jpg



1, 2, 3, 4 = SC1 primers
1,2 = AlwI
3,4 = NBI
5 = neg control (no template) (which primers not clear -> ask sinisha)
15 = pos control

RPA_2 Pyrophosphate detection

Project: Lab notebook
Authors: Marijn van den Brink
Created at: 2020-08-04T13:12:00.207220+00:00

TUESDAY, 4/8/2020

Dilutions of pyrophosphate (PPi) were made and the absorbance was measured at 600 nm in presence of the pyrophosphate detection reagents (as described in protocol RPA_2).

Plate (numbers refer to concentration PPi in µM):

Well1

	1	2	3	4	5	6	7	8	9	10	11	12
A	40	40	40									
B	60	60	60									
C	80	80	80									
D	100	100	100									
E												
F	Neg control											
G												
H												

Results:

RPA_2 PPi concentrations 100 80 60 40 uM.xlsx

RPA_2_PPi_concentrations_100_80_60_40_uM calib curve graph.xlsx

The absorbance of PDADMAC slightly decreases with an increasing concentration of PPi, but the variation in the triplicates is too large to validate this. Instead of 600 nm, PDADMC could also be measured at 550 nm in a follow-up experiment.
Try on LAMP products anyway.

RPA Kinetic Measurements

Project: Lab notebook
Authors: Tim van den Akker
Created at: 2020-08-05T19:28:39.293762+00:00

WEDNESDAY, 5/8/2020

Methods

Followed protocol RPA_4 Kinetic Measurement protocol at temperatures 4, 20, 25 and 30 degrees as is shown in Table 1,; for each temperature 4 samples were used to determine the DNA concentration at 4 different timepoints during the incubation step (using 2 different Nanodrop devices and gel electrophoresis)

- There was a fault in the machine settings when starting the measurements for 20 degrees, as the reaction starts after the addition of MgOAc, right before incubation, these kinetic measurements for 20 degrees will be redone.
- As there was not enough genomic yeast DNA for the 37 degrees measurements that are described in the protocol, these will also be performed later when additional template is produced/delivered
- As the 4 degrees measurement was just meant to check whether the reaction was active at this temperature, only one sample was used to measure after 20 min. of incubation.
- For all samples, the SC1 forward and SC1 Nt.AlwI reverse primer were used as these showed the best results on the gel of 4-8-2020.
- The negative controls contained the same primers as the sample but no template, the positive control was performed with the DNA and primer mix from the TwistAmp Basic kit from TwistDX.

Gel electrophoresis was performed with a 1.5% agarose gel that was run for 30 minutes with 5µL of each sample mixed with 1µL of Loading buffer, to check whether some of the results found on the gel were due to pipetting errors, the gel will be rerun on 6-8-2020.

Nanodrop Results

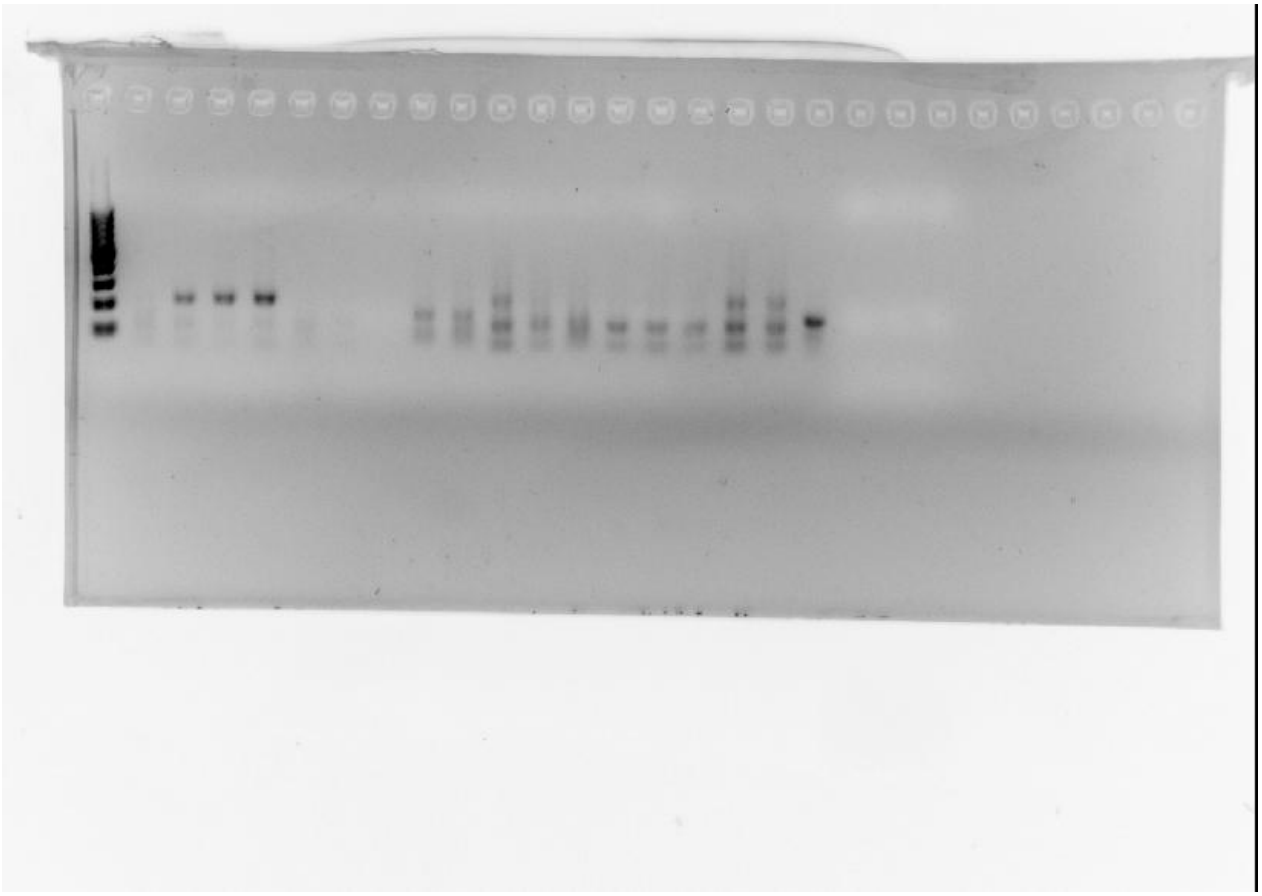
Table1

	A	B	C	D	E	F	G	H	I	J	K	L	M
1	**Temperatures (degrees Celcius)**	4		Average	20		Average	25		Average	30		Average
2	**Concentration after 5 min. (ng/µL)**	-	-	-	30.0	30.6	30.3	11.4	10.1	10.75	30.4	28.7	29.55
3	**Concentration after 10 min. (ng/µL)**	-	-	-	37.0	35.2	36.1	23.7	24.4	24.05	30.6	28.5	29.55
4	**Concentration after 15 min. (ng/µL)**	-	-	-	42.7	40.0	41.35	30.4	29.2	29.8	43.3	41.3	42.3
5	**Concentration after 20 min. (ng/µL)**	18.3	17.8	18.05	37.9	36.9	37.4	27.2	26.0	26.6	46.9	40.6	43.75
6	**Negative control**	22.4	21.0	21.7	22.0	20.8	21.4	28.3	27.1	27.7	41.4	38.3	39.85
7	**Positive control**	13.4	13.4	13.4	33.4	32.5	32.95	27.1	26.1	26.6	40.4	37.7	39.05

Gel Electrophoresis Results (see below for gel)

- The bands for 10-20 min. samples for 20 degrees (lane 3-5) showed clear bands, which, as expected show between 200 and 250bp (the amplicon should be 219bp), but for reasons mentioned previously, these results might be negatively affected by experimental errors.
- The remaining lanes showed no or vague bands, therefore the gel will be rerun on 6-8-2020 to check whether this was due to pipetting errors or maybe the result of unspecific binding and/or primer dimers.

docent 2020-08-05 18hr 43min.jpg



PDADMAC Measurements

Project: Lab notebook
Authors: Tim van den Akker
Created at: 2020-08-06T13:30:51.091610+00:00

THURSDAY, 6/8/2020

Protocol RPA_2.

Methods

- The Tecan platereader was used to check the intensity of the color change (pink > purple), time was not a factor.
- The solutions in the 384 well plate were as follows:

Table1

	A	B	C	D	E	F	G
1	**D1**	**D2**	**D3**	**D4**	**D5**	**D6**	**D7**
2	PDADMAC	PDADMAC + RPA sample	PDADMAC + RPA sample	PDADMAC + PPi solution (40µM)	PDADMAC + PPi solution (60µM)	PDADMAC + PPi solution (80µM)	PDADMAC + PPi solution (100µM)

- The RPA sample used was prepared on 5-8-2020 without the cleanup step
- In the attached excel files, the measurements of interest, located in D1-7 of the table are marked in orange

Results

PDADMAC RPA + PPi after 5 minutes.xlsx

PDADMAC RPA + PPi in double ratio PDADMAC_sample at 550nm.xlsx

PDADMAC RPA + PPi in double ratio PDADMAC_sample.xlsx

PDADMAC RPA + PPi kinetic loop.xlsx

Observations:

- There were bubbles in well 2: only look at well 3 for PDADMAC+RPA

--> If repeated:

- Change the 384 well plate: the volume was too small for the plate --> either diluted/put in buffer, try 96?
- Calibration curve for PPi: should be more dilutions, and should be less diluted: PPi values< than RPA outcome
- Measure at 550 nm and 600 nm
- Duplicates for the calibration curve and triplicated for the RPA

RPA Kinetic Measurements (2)

Project: Lab notebook
Authors: Tim van den Akker
Created at: 2020-08-06T13:09:46.979958+00:00

THURSDAY, 6/8/2020

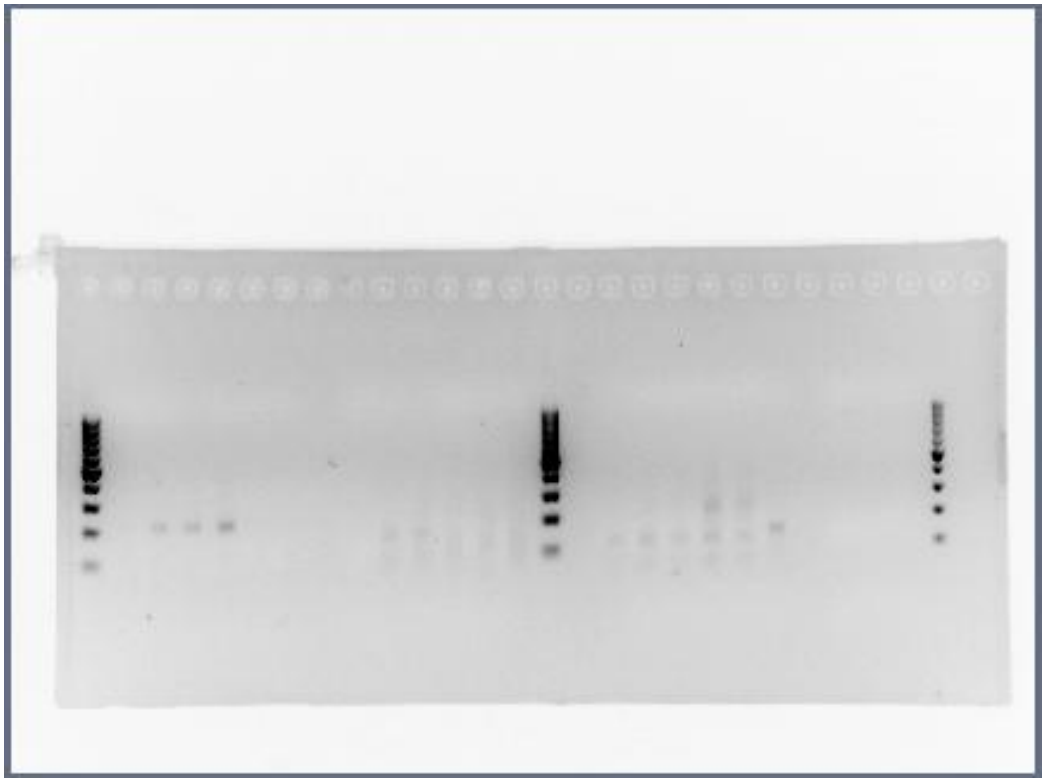
Methods

- Made a another gel with the kinetic measurement samples from 5-8-2020, as the absence of bands in several of the lanes might have been due to pipetting errors.

Gel Electrophoresis Results

- As expected the samples incubated at 4 degrees did not show any results
- The samples measured at 20 degrees again showed bands at the 10 till 20 min. incubation, but the bands are less clear.
- The samples measured at 25 and 30 degrees show a yet unexplained pattern, which could be unspecific binding, primer dimers or contamination which the primers could have reacted with.
- This gel shows results that are similar to the ones on the gel of 5-8-2020, however, the bands appear less clear

2020-08-06 RPA kinetics redo.jpg



RPA Kinetic Measurements (3)

Project: Lab notebook
Authors: Tim van den Akker
Created at: 2020-08-08T12:15:35.195467+00:00

FRIDAY, 7/8/2020

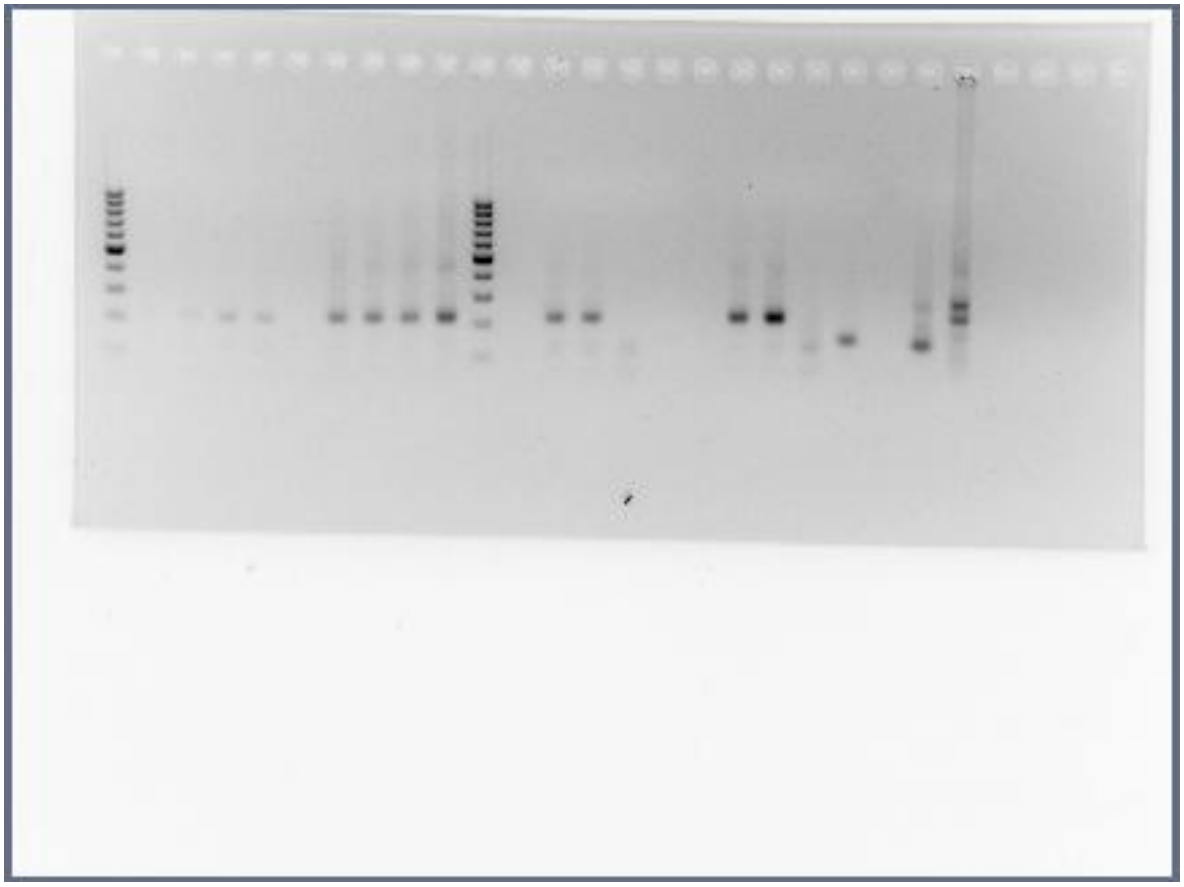
Methods

- Reran the endpoints for each temperature: 20, 25, 30 and 4 degrees on a gel
- Made samples for both 20 and 42 degrees according to RPA_4 Kinetic Measurement protocol to recheck 20 degrees and check 42 degrees
- Nanodrop and gel results will be performed on 10-8-2020.

Results

- For 20 degrees (lanes 2-5) the bands gradually become more clear as the amount of DNA is higher in each of the samples from the successive timepoints, which is as expected.
- For 42 degrees (lanes 7-10) the bands show a similar pattern but with less variation, as expected based on the recommendation on the kit for the temperature used.
- The duplicates of the endpoints for 25 and 30 degrees (lanes 13+14 and 18+19) and their negative (lanes 15 and 20) and positive (lanes 16 and 21) controls show clear bands
- The bands in the final two lanes (23 and 24) are from a different experiment.

10-8-2020 RPA kinetics 20 (4), 42 (4), 25 (2xend, n, p), 30 (2xend, n, p).jpg



- The Nanodrop showed the following results for the samples used in the gel above

Table1

	A	B	C	D	E
1	**Temperature**	20	42	25	30
2	**Concentration after 5 min. (ng/µL)**	8.5	39.0	-	-
3	**Concentration after 10 min. (ng/µL)**	16.0	36.9	-	-
4	**Concentration after 15 min. (ng/µL)**	16.4	43.4	-	-
5	**Concentration after 20 min. (ng/µL)**	15.8	50.0	43.7/39.2*	40.5/46.7*
6	**Negative control**	-	-	37.2	32.8
7	**Positive control**	-	-	30.2	36.0

*As stated above, the 25 and 30 degrees samples were only measured at the endpoint (after 20 min.) in duplicate

RT-qRPA Kinetic Measurements

Project: Lab notebook
Authors: Tim van den Akker
Created at: 2020-08-11T09:42:20.307122+00:00

MONDAY, 10/8/2020

Method

- Run RPA samples with two different primer sets at 42 degrees in a qPCR machine using SYBR Green as an alternative to the previous kinetic measurements taken using a regular PCR machine.
    - SC1_F_p1 forward primer was used in combination with either the SC1_Nt.AlwI or SC1_Nt.BstNBI reverse primer
    - Run included one postive control and two negative controls; one for each primer set
    - The machine measured every 30 sec. for 40 times.
    - For each mixture (sample, pc and nc) 2µL of RNase/RNase free water was replaced with 25x SYBR Green; all the other components were added in the same amounts/concentrations
- Allows us to take more regular and precise measurements without the need to take samples out which could disrupt other samples in the machine

Table1

	A	B	C	D	E
1	**A1**	**A2**	**A3**	**A4**	**A5**
2	SC1_F_p1 and SC1_R_Nt.AlwI	SC1_F_p1 and SC1_R_Nt.BstNBI	positive control	Negative control for A1	Negative control for A2

Results

- Due to a too low concentration of SYBR Green (625x dilution instead of 25x dilution), the signals were weaker than the background fluorescence and could not be measured. Will be redone on 11-8-2020. The results of the qPCR are therefore not included.

RT-qRPA Kinetic Measurements (2)

Project: Lab notebook
Authors: Tim van den Akker
Created at: 2020-08-11T14:26:54.631472+00:00

TUESDAY, 11/8/2020

Method

- Run RPA samples with two different primer sets at 42 degrees in a qPCR machine using SYBR Green as an alternative to the previous kinetic measurements taken using a regular PCR machine.
  - SC1_F_p1 forward primer was used in combination with either the SC1_Nt.AlwI or SC1_Nt.BstNBI reverse primer
  - Run included one postive control and two negative controls; one for each primer set
  - The machine measured every 30 sec. for 40 times.
  - For each mixture (sample, pc and nc) 2µL of RNase/RNase free water was replaced with 25x SYBR Green; all the other components were added in the same amounts/concentrations
- Allows us to take more regular and precise measurements without the need to take samples out which could disrupt other samples in the machine

Table1

	A	B	C	D	E
1	**F1**	**F2**	**F3**	**F4**	**F5**
2	SC1_F_p1 and SC1_R_Nt.AlwI	SC1_F_p1 and SC1_R_Nt.BstNBI	positive control	Negative control for A1	Negative control for A2

- The same procedure was repeated with at 37 degrees with the following changes:
  - Only the SC_F_p1 forward primer and SC1_R_Nt.AlwI reverse primer were used in triplicate with a positive control

Table2

	A	B	C	D
1	**D1**	**D2**	**D3**	**D4**
2	SC1_F_p1 and SC1_R_Nt.BstNBI	SC1_F_p1 and SC1_R_Nt.BstNBI	SC1_F_p1 and SC1_R_Nt.BstNBI	Positive control

Results

- For 42 degrees

Res1_11082020 - End Point Results.xlsx

Res1_11082020 - Melt Curve Plate View Results.xlsx

Res1_11082020 - Quantification_Amplification_Results (with graph).xlsx

Res1_11082020 - Quantification Cq Results.xlsx

Res1_11082020 - Quantification Plate View Results.xlsx

Res1_11082020 - Quantification Summary.xlsx

Res1_11082020 - Standard Curve Results.xlsx

- For 37 degrees

1182020_37 - End Point Results.xlsx

1182020_37 - Melt Curve Plate View Results.xlsx

1182020_37 - Quantification Amplification Results (with graph).xlsx

1182020_37 - Quantification Cq Results.xlsx

1182020_37 - Quantification Plate View Results.xlsx

1182020_37 - Quantification Summary.xlsx

1182020_37 - Standard Curve Results.xlsx

Threshold of detection of RPA

Project: Lab notebook
Authors: Violette Defourt
Created at: 2020-08-11T14:24:29.663671+00:00

TUESDAY, 11/8/2020

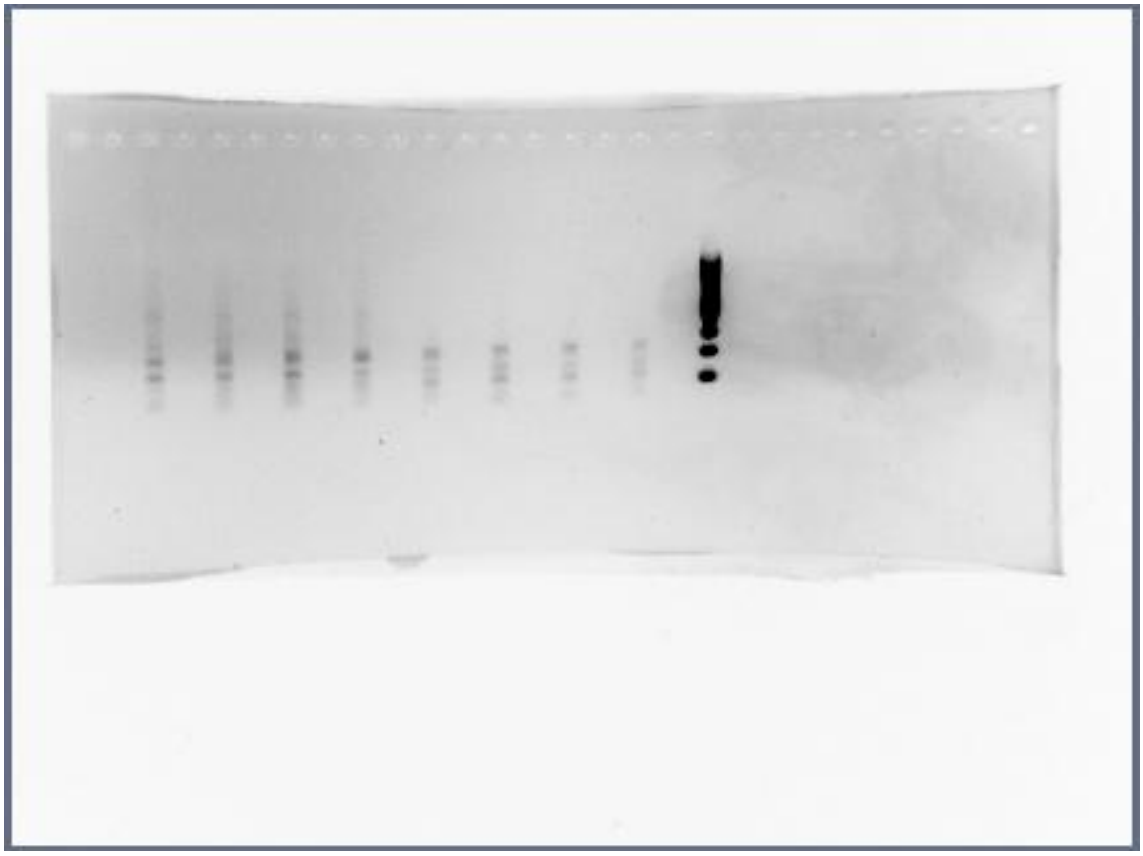
Methods

The LOD of RPA was determined using serial dilutions of template of a known calculated concentration (- - - - -). The outcome of the RPA reaction at 42°C for 20 minutes and after cleanupwas determined using gel electrophoresis and Nanodrop.

Note: the same serial dilutions were used qRT-RPA!

Result from the gel electrophoresis:

11-08-20 Gel RPA LOD.jpg



From left to right:
Equivalent (copies/micoL): 11,06 x 10^7 - 11,06 x 10^6 - 11,06 x 10^5 - 11,06 x 10^4 - 11,06 x 10^3 - 11,06 x 10^2 - 11,06 x 10 - 11,06 - DNA ladder

Data from the Nanodrop measurements:

Table1

	A	B	C	D	E	F	G
1	Results from the Nanodrop measurement						
2		Concentration (copies/microL)	Nucleic acid (ng/microL)	A260	A280	260/280	260/230
3	1	1.11e+8	37.2	0.744	0.317	2.35	2.29
4			37.4	0.748	0.325	2.3	2.35
5	2	1.11e+7	38.8	0.775	0.328	2.37	2.28
6	3	1.11e+6	38.4	0.767	0.33	2.232	2.22
7	4	1.11e+5	44.2	0.885	0.374	2.37	2.19
8			43.8	0.875	0.387	2.26	2.2
9	5	1.11e+4	38.1	0.763	0.312	2.44	2.04
10	6	1.11e+3	40	0.8	0.317	2.53	2.26
11	7	1.11e+2	28.7	0.574	0.255	2.25	2.01
12	8	1.11e+1	30.3	0.607	0.251	2.42	2.04
13			28.8	0.576	0.21	2.74	2.13

ANALYSIS:

RPA works in all the wells! So the limit of detection is of only a few copies in total :)

--> perhaps we could even try 1,106 copies? --> qPCR

RT-qRPA Kinetic Measurements (3)

Project: Lab notebook
Authors: Tim van den Akker
Created at: 2020-08-12T13:57:33.584296+00:00

WEDNESDAY, 12/8/2020

Method

- Run RPA samples with two different primer sets at 30 degrees in a qPCR machine using SYBR Green as an alternative to the previous kinetic measurements taken using a regular PCR machine.
  - SC1_F_p1 forward primer and the SC1_Nt.BstNBI reverse primer were used in triplicate with a positive control
  - Run included one postive control
  - The machine measured every 30 sec. for 40 times.
  - For each mixture (sample, pc and nc) 2µL of RNase/RNase free water was replaced with 25x SYBR Green; all the other components were added in the same amounts/concentrations
- Allows us to take more regular and precise measurements without the need to take samples out which could disrupt other samples in the machine

Table2

	A	B	C	D
1	**G1**	**G2**	**G3**	**G4**
2	SC1_F_p1 and SC1_R_Nt.BstNBI	SC1_F_p1 and SC1_R_Nt.BstNBI	SC1_F_p1 and SC1_R_Nt.BstNBI	Positive control

Results

- For 30 degrees

12082020_30degC - End Point Results.xlsx

12082020_30degC - Melt Curve Plate View Results.xlsx

12082020_30degC - Quantification Amplification Results (with graph).xlsx

12082020_30degC - Quantification Cq Results.xlsx

12082020_30degC - Quantification Plate View Results.xlsx

12082020_30degC - Quantification Summary.xlsx

12082020_30degC - Standard Curve Results.xlsx

Discussion

- Based on the graph made using the '12082020_30degC - Quantification Amplification Results (with graph).xlsx' file, the starting concentration of template used for the samples was too high to properly compare it to the positive control. For other runs at the remaining temperatures, a lower concentration of the template will be used.

Threshold of detection RPA

Project: Lab notebook
Authors: Violette Defourt
Created at: 2020-08-12T10:17:28.793862+00:00

WEDNESDAY, 12/8/2020

We repeated the experiment from 11/08, with more lower dilutions to get the window of detection. Therefore, we once again used the stock solution (53,1 ng/microL) and diluted it tenfold until a dilution of 11,06 x 10^-3. We also tried 1/2 steps at x10^1 and 10^0, in case the detection would differ at the lower concentrations.

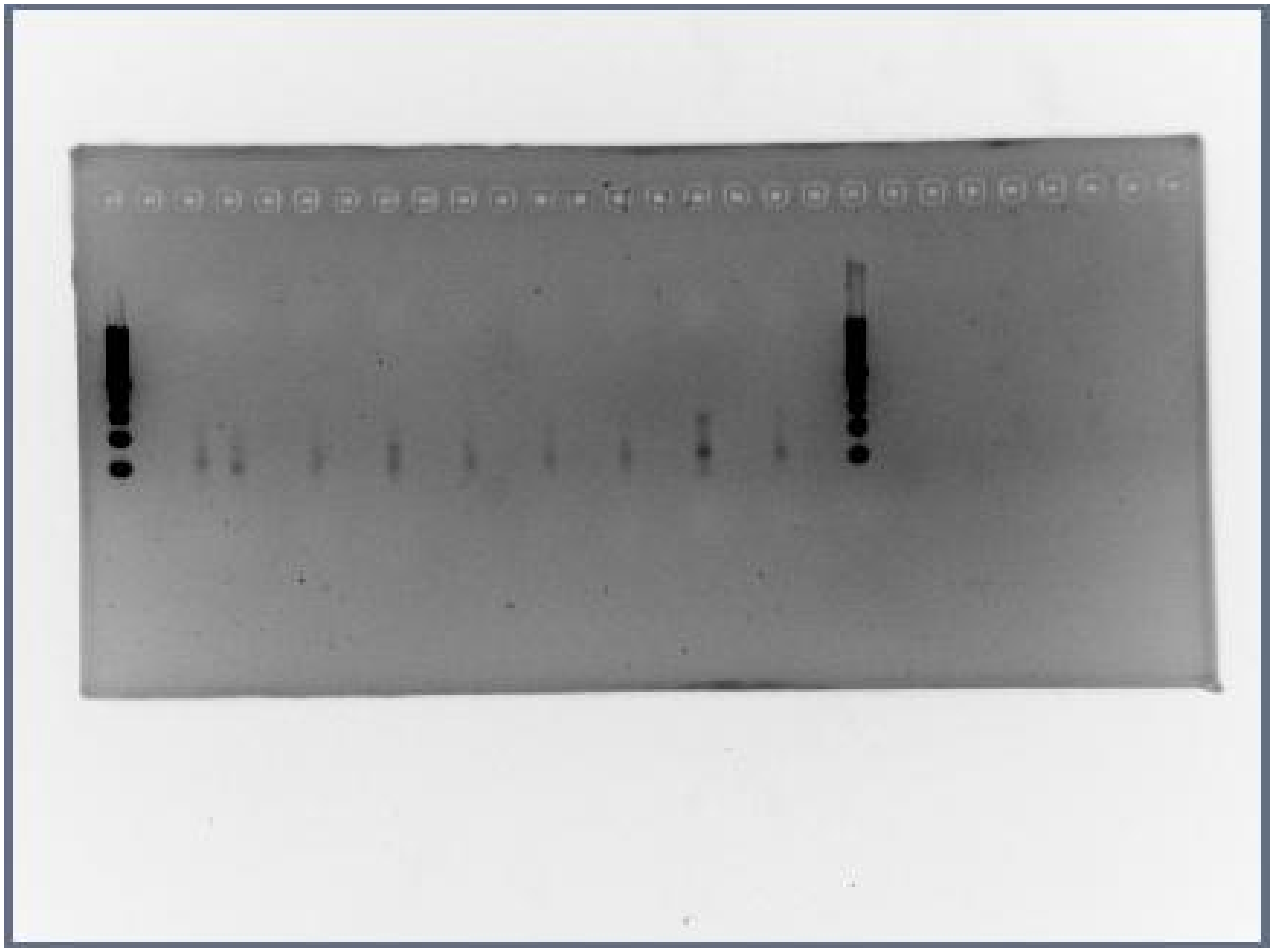
Diutions:

Table1

	A	B
1	Sample	Copies (total for 5 microL sample)
2	1	1106
3	2	110,6
4	3	55,31
5	4	11,06
6	5	5,531
7	6	1,106
8	7	0,106
9	8	0,0106
10	9	0

We had a negative control this time, and tested it on the Nanodrop and it showed some 40ish ng/microL
--> Therefore we didn't bother testing the other samples and instead ran it on a gel:

12-08-20 Gel RPA LOD.2sc.jpg



Samples order: 1 to 8 and negative control
--> Negative control shows presence of a vague band
This gel is crappy
--> we have to try qRPA

RT-qRPA Template Concentration Gradient

Project: Lab notebook
Authors: Tim van den Akker
Created at: 2020-08-13T14:45:30.246882+00:00

THURSDAY, 13/8/2020

Method

- Run RPA samples with two different primer sets at 42 degrees in a qPCR machine using SYBR Green as an alternative to the previous kinetic measurements taken using a regular PCR machine.
  - SC1_F_p1 forward primer and the SC1_Nt.BstNBI reverse primer were used in combination with different template concentrations
  - The machine measured every 30 sec. for 40 times.
  - For each mixture/sample, 2µL of RNase/RNase free water was replaced with 25x SYBR Green; all the other components were added in the same amounts/concentrations
  - No negative or positive control was included
- Allows us to take more regular and precise measurements without the need to take samples out which could disrupt other samples in the machine
- The goal was to test the sensitivity of the RPA through the use of different template concentrations which would represent different amounts of pathogen units in a sample.

Table2

	A	B	C	D
1	**H1**	**H2**	**H3**	**H4**
2	SC1_F_p1 and SC1_R_Nt.BstNBI + 1106 copies template	SC1_F_p1 and SC1_R_Nt.BstNBI + 110,6 copies templa	SC1_F_p1 and SC1_R_Nt.BstNBI + 11,06 copies templa	SC1_F_p1 and SC1_R_Nt.BstNBI + 1,106 copies templa

Results

1382020_42 - End Point Results.xlsx

1382020_42 - Melt Curve Plate View Results.xlsx

1382020_42 - Quantification Amplification Results (with graph).xlsx

1382020_42 - Quantification Cq Results.xlsx

1382020_42 - Quantification Plate View Results.xlsx

1382020_42 - Quantification Summary.xlsx

1382020_42 - Standard Curve Results.xlsx

- As we forgot to add SYBR Green to the sample in H3, the graph made from the measurements of this sample appears flat.

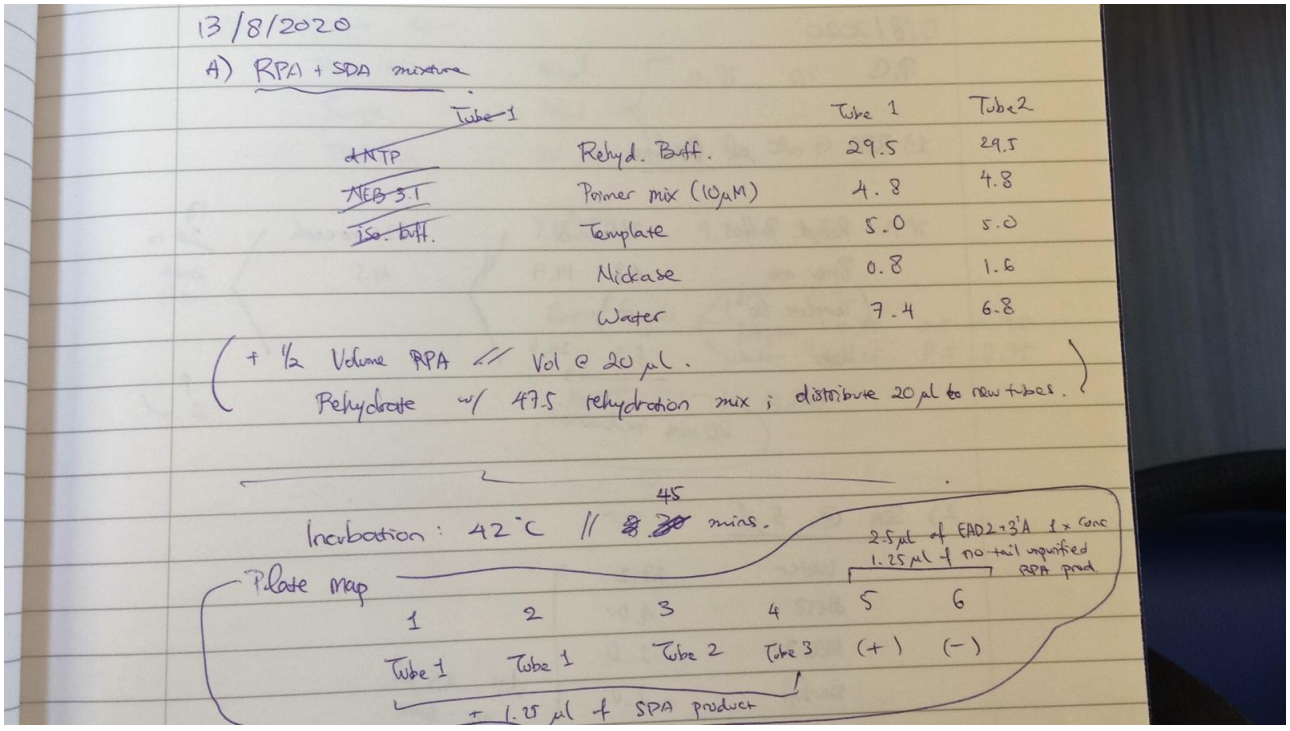
Integration RPA and nickase

Project: Lab notebook
Authors: Marijn van den Brink
Created at: 2020-08-14T05:19:14.577010+00:00

THURSDAY, 13/8/2020

This experiment was performed as an attempt to combine the RPA and SDA reaction into a one-pot reaction. Therefore, the nickase was added to the RPA mixture.

image.png



Integration RPA and SDA STOP.xlsx

Integration RPA and SDA.xlsx

RPA+SDA_Products _0.75 0.5 0.2_x2 + PC x3 + background without DTT_STOP.xlsx

RPA+SDA_Products _0.75 0.5 0.2_x2 + PC x3 + background without DTT.xlsx

WrongWellsorSamples_non-purifiedRPA-SDA-GQ_STOP.xlsx

RPA negative controls: primer screening

Project: Lab notebook
Authors: Marijn van den Brink
Created at: 2020-08-17T14:16:02.064057+00:00

MONDAY, 17/8/2020

On August 14, we discovered that the proof of concept (RPA - SDA - GQ oxidation) also leads to TMB oxidation when there was no target added to the RPA reaction. We hypothesize this could be the result of any amplification of the primers, primer dimers or other secondary structures. To screen for primers that do not amplify by themselves or form secondary structures, we perform RPA negative controls with multiple primer sets.

Primer sets:
Plasmodium falciparum:

- forward: PF1_F
- reverse: PF1_R_AlwI and PF1_R_NBI

Mycobacterium:

- forward: MB1_F
- reverse: MB1_R_AlwI and MB1_R_NBI

Saccharomyces cerevisiae:

- forward: SC3_F
- reverse: SC3_R_AlwI and SC3_R_NBI

- forward: SC4_F
- reverse: SC4_R_AlwI

Influenza A:

- forward: IA1_F
- reverse: IA1_R_AlwI and IA1_R_NBI

15 µL raw RPA product was transferred to a new tube and purified. The residual unpurified RPA product was used for subsequent SDA and GQ oxidation reactions.

7x Nt.BstNBI premix was made:

Table1

	A	B	C
1	Water	46.41	µL
2	dNTP	7	µL
3	NEBuffer 3.1	3.5	µL
4	Isothermal amplification buffer	3.5	µL
5	Bst 2.0 DNA polymerase	0.35	µL
6	Nt.BstNBI	2.24	µL
7	**Total volume:**	**63**	**µL**

RPA reactions:

Table2

	A	B	C	D	E	F	G	H	I	J	K	L	M
1	Tube#	1	2	3	4	5	6	7	8	9	10	11	12
2	Forward primer	PF1	PF1	PF2	PF2	MB1	MB1	SC3	SC3	SC4	IA1	IA1	SC1
3	Reverse primer	AlwI	NBI	AlwI	NBI	AlwI	NBI	AlwI	NBI	AlwI	AlwI	NBI	NBI

SDA reactions:

Table3

	A	B	C	D	E	F	G	H	I	J	K	L	M
1	Tube#	1	2	3	4	5	6	7					
2	Template (RPA product)	2 (PF1 NBI)	4 (PF2 NBI)	6 (MB1 NBI)	8 (SC3 NBI)	11 (IA1 NBI)	12 (SC1 forward primer and SC4 reverse primer NBI)	Purified RPA product (NBI) (from proof of concept last wednesday and thursday experiments)					
3													

SDA reactions were incubated at 55 degrees for 40 minutes.

For tube 1-4, 2.2 µL RPA product was pipetted into the SDA premix instead of the usual 1 µL. To compensate for that, 0.612 µL SDA product was transferred to the oxidation reaction instead of 1.25 µL.
For tube 5-7, 1 µL RPa product was pipetted into the SDA premix, and later 1.25 µL SDA product was transferred to the oxidation reaction.

Oxidation reactions:

Well1

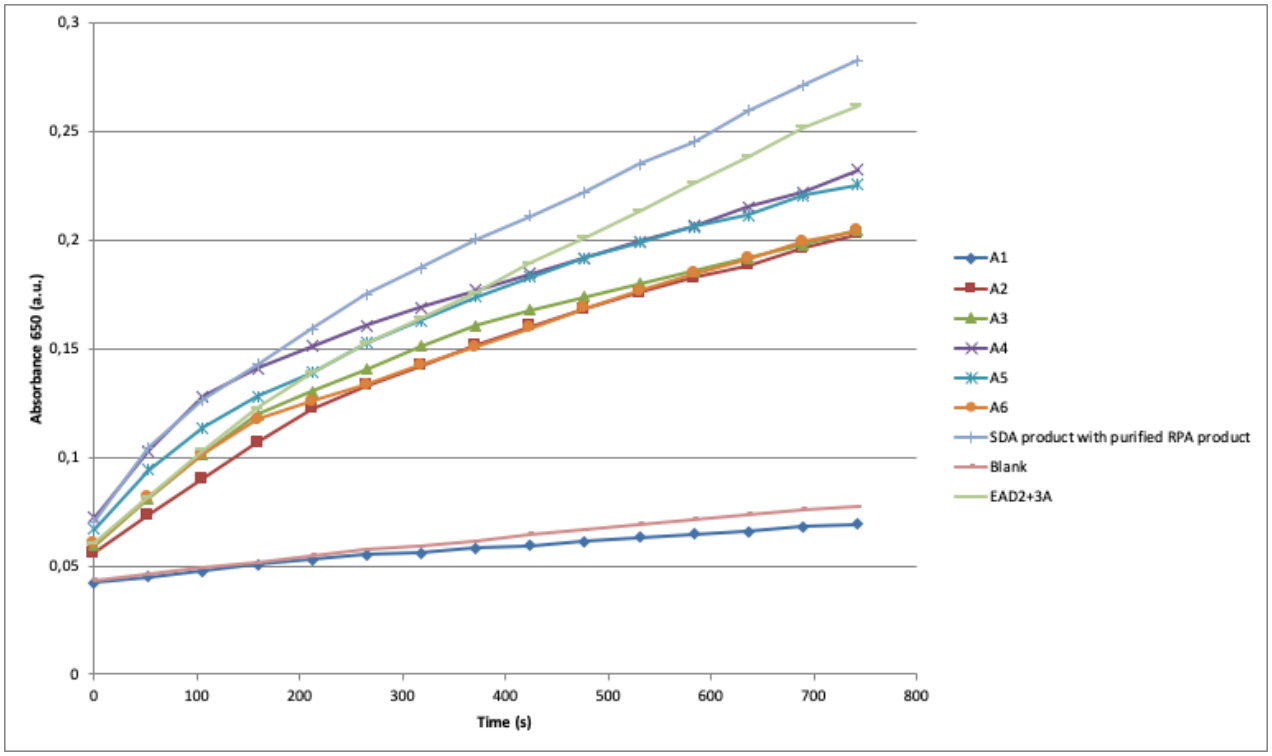
	1	2	3	4	5	6	7	8	9	10	11	12
A	SDA product PF1 NBI	SDA product PF2 NBI	SDA product MB1 NBI	SDA product SC3 NBI	SDA product AI1 NBI	SDA product SC1/4 NBI	SDA product purified RPA (NBI)	BLANK (PCB pH 3.8)	EAD2 +3A			
B												
C												
D												
E												
F												
G												
H												

Results:
Gel electrophoresis:
RPA product was purified and loaded on agarose gel. No bands were observed for all reactions.

Nanodrop:
yields between ~20 and ~40 ng/µL were observed.

Oxidation:
The oxidation reaction of primer set PF1 (Nt.BstNBI) showed a similar absorbance curve at 650 nm as the negative control. All other reactions had similar absorbance curves at 650 nm to the positive control (EAD2+3A). This means that all the primer sets used in this experiment, except PF1 (Nt.BstNBI), in some way (e.g. primer amplification, primer dimer, secondary structures) lead to a colour change in the oxidation in the absence of RPA target sequence. Therefore, PF1 (Nt.BstNBI and maybe AlwI too) may be a potential target for our diagnostic tool.

Aug 17 Negative controls RPA.png



Negative RPA controls 2nd try STOP.xlsx

Negative RPA controls 2nd try.xlsx

7 times SDA mixture with NBI was made

Table5

	A	B
1	Component	amount
2	Water	40.47
3	10 mM dNTP	6
4	NEB buffer 3.1	3
5	Isothermal amplification Buffer	3
6	Bst 2.0 DNA Pol	0.30
7	Nt.BstNBI nickase	0.96
8	Total	54

False Positive Troubleshooting #1

Project: Lab notebook

Authors: Sebastian Tommi Tandar

Created at: 2020-08-19T15:35:37.038349+00:00

FRIDAY, 14/8/2020

RAW DATA MISSING!

Objective is to pinpoint the source of false positive signal.

Negatives Troubleshooting_RPA+SDAprod niRPAblank RPAkitOnly PrimerOnly(noDTT) EADin_niRPAblank STOP.xlsx

Negatives Troubleshooting_RPA+SDAprod niRPAblank RPAkitOnly PrimerOnly(noDTT) EADin_niRPAblank.xlsx

Negatives Troubleshooting_RPAprod niRPAblank RPAkitOnly PrimerOnly EADin_niRPAblank.xlsx

6 conditions were checked:

1. Blank RPA to SDA product (for comparison)
2. Blank RPA (non-incubated) used for SDA (identifies the importance of RPA reaction)
3. Blank RPA (non-incubated) used for SDA; spiked with EAD2+3'A (to identify the signal when GQ DNAzyme is produced)
4. RPA mix only used for SDA (to identify possible signal sources from RPA mix)
5. Primer only passed to SDA (to identify possible signal from primer alone)
6. Primer used immediately for GQ DNAzyme reaction - taken over by Marijn on a separate experiment

Plate Map:

Table1

	A	B	C	D	E	F	G	H	I	J	K	L
1	(1)	(1)	(2)	(2)	(4)	(4)	(5)	(5)	(3)	(3)		

Result:

all 2-6 did not give a positive signal.

Thus, PRIMERS and RPA incubation were both important. >> CHECK IF SDA INCUBATION IS ESSENTIAL

Running Hyp: inspecific primer-primer interaction mediates GQ production in the absence of target.

RPA on Saccharomyces genome

Project: Lab notebook
Authors: Marijn van den Brink
Created at: 2020-08-17T20:31:26.870580+00:00

MONDAY, 17/8/2020

We discovered that we have been doing RPA with the wrong primers for some time (at least August 10 to 14). SC1 forward primer was combined with the SC4 reverse primer (Nt.BstNBI).

To check again with the right primer set for SC1, new Saccharomyces cerevisiae genome was obtained (Marjolein), two genomic samples were given to us, of an unknown concentration and that may have been damaged using DNAse.

The concentration was determined/approximated using Nanodrop:

Table1

	A	B	C	D	E	F
1		Concentration (ng/microL)	A260	A280	280/260	260/230
2	Template 1	82,8	1,656	0,843	1,96	1,52
3	Template 2	36,6	0,732	0,406	1,80	1,18

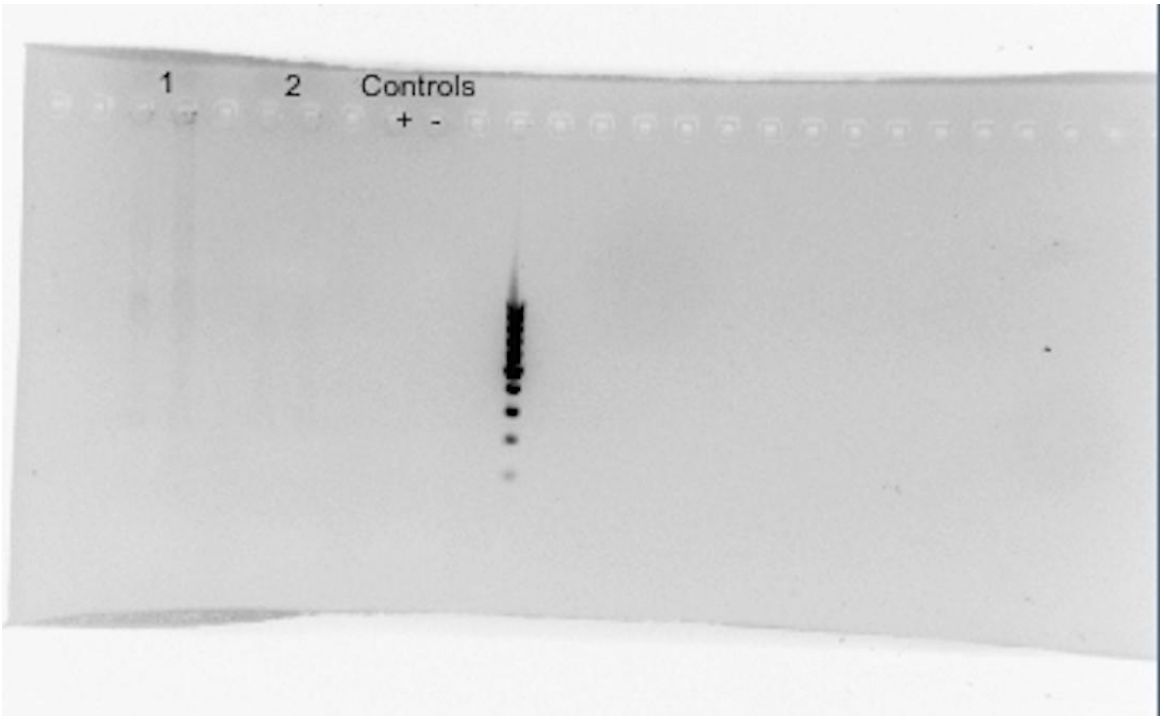
The RPA was done in duplo, in half-doses of RPA reagent, with a positive and negative control and put on a gel in the following order:
Note: the samples were not purified after amplification as there were not enough purification columns and Marjolein said that we should still be able to detect something.

Table2

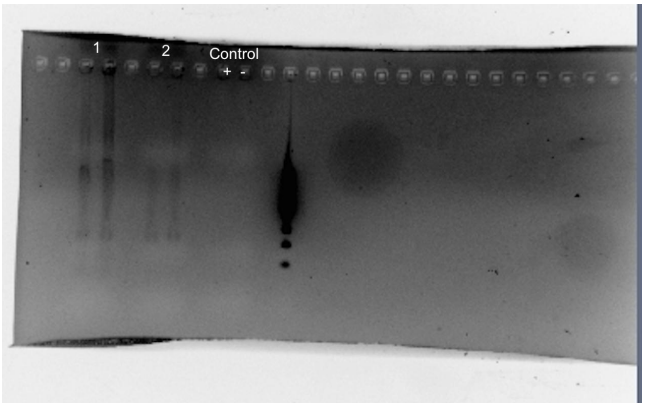
	A	B	C	D	E	F
1	Template 1	Template 1	Template 2	Template 2	Positive control	Negative control

7 microL of sample was added to the gels

RPA new templates with right primers 2.jpg



RPA new templates with right primers.jpg



The samples indicated faint bands --> since they are smears, the samples are probably indeed quite digested but it seems like at two places there are faint bands (don't know what they stand for? could the lower one be our template amplicon?). The smears were due to the absence of the purification step.
Also, I have no clue why the positive control is not positive (I am very certain I have put in the template and the primers in the right amounts). Sebastian mentioned he already had the same once... I don't know whether it could be a matter of template..? --> This could also be due to no purification

Follow-Up: RPA False-Positive Troubleshooting with RT-RPA (SYBR Green)

Project: Lab notebook
Authors: Sebastian Tommi Tandar
Created at: 2020-08-21T19:42:49.579172+00:00

THURSDAY, 20/8/2020

Basically to confirm results obtained from: RPA False-Positive troubleshooting performed on the same day. Here we aim to confirm that:

1. The forward primer was indeed the culprit for the false-positive signal (somehow)
2. DMSO concentration can be tuned such that false-positive signals were silenced without eliminating true-positive signals

DMSO concentrations evaluated: 10%, 5%, and 2.5% in the final RPA mixture.

PlateMap.xlsx

admin_2020-08-20 15-55-41_CT017461 - Quantification Amplification Results.xlsx

CONCLUSIONS

1. Despite not having a good explanation for this, the forward primer of SC1 primer set was indeed having a self-amplification tendency. However, it might not produce the positive signal without the reverse primer - although this needs to be confirmed by a separate experiment.
2. True-positive signal can be selected by reducing DMSO concentration. 2.5% DMSO concentration allowed true-positive signal to reach its maxima within ~20 cycles while still silencing the false-positive signal.

RPA False-Positive Troubleshooting with RT-RPA (SYBR Green)

Project: Lab notebook
Authors: Sebastian Tommi Tandar
Created at: 2020-08-21T19:26:45.523919+00:00

THURSDAY, 20/8/2020

This experiment was performed to identify the factor that caused high false-positive signals during RPA. Template amplification was monitored in a real-time manner using qPCR machine (BioRad) and by using SYBR Green dye (25x concentration used; 2.5 uL added to a 50 mL reaction premix) as dsDNA probe. Synthetic *S. cerevisiae* template (~600 bp) was used as a "target" for the amplification. Reactions were performed at a 20 uL scale. 1 uL of MgOAc was pipetted to the bottom of each well. The reaction was started by pipetting RPA premixes to its corresponding wells using a multipipette. The following scenarios were investigated:

1. Template (+) in the presence of F-primer and NtBstNBI-tailed R-primer
2. Template (+) in the presence of F-primer and non-tailed R-primer
3. Template (-) F-primer
4. Template (-) NtBstNBI-tailed R-primer
5. Template (+) in the presence of F-primer and NtAlwI-tailed R-primer
6. Template (+) in the presence of F-primer and NtAlwI-tailed R-primer; both primers at half concentration
7. Template (-) in the presence of F-primer and NtAlwI-tailed R-primer
8. Template (-) in the presence of F-primer and NtAlwI-tailed R-primer; both primers at half concentration
9. Template (-) in the presence of F-primer and NtBstNBI-tailed R-primer in with 10% DMSO concentration

PlateMap.xlsx

admin_2020-08-20 10-48-28_CT017461 - Quantification Amplification Results.xlsx

CONCLUSION

a) Result from scenarios 3 and 4: problem was caused mainly by F-primer. Tailed R-primer never give rise to unspecific amplification. (DOUBLE-CHECKED!)

b) Result from scenario 9: DMSO (at 10% final concentration) can alleviate primer-primer interactions. However, at this concentration, primer-target interactions were also eliminated. **Try to find a lower DMSO concentration that eliminates primer-primer interactions but enough to fully silence primer-target signals.**

Confirming DMSO Effects on RPA - Lower DMSO Concentrations

Project: Lab notebook
Authors: Sebastian Tommi Tandar
Created at: 2020-08-21T19:59:53.620238+00:00

FRIDAY, 21/8/2020

Here we evaluated the effects of lower DMSO concentrations on RPA specificity and occurence of background noise when the reaction was performed in the absence of templates. DMSO concentrations investigated were 2.5%, 1.0%, and 0.5% in the final reaction mix.

Overall trend:
No template controls always shown significantly lower signal production in exception to 2.5% DMSO. This might be due to well mix-up, or improper pipetting (indeed, a lot of bubbles were formed in this experimental replicate. Re-experimentation is advised.

DMSO concentrations of lower than 2.5% allowed increased background signal production. DMSO concentrations of at least 2.5% was thus advised. **Higher concentrations (but below 5.0%) might be suitable.**

PlateMap.xlsx

admin_2020-08-21 10-58-28_CT017461 - Quantification Amplification Results.xlsx

admin_2020-08-21 10-58-28_CT017461 - Melt Curve Derivative Results.xlsx

Evaluating the Effects of Higher DMSO Concentrations on RPA

Project: Lab notebook
Authors: Sebastian Tommi Tandar
Created at: 2020-08-21T20:06:06.173635+00:00

FRIDAY, 21/8/2020

Here we tried to further increase DMSO concentration in RPA reactions to 3.0%, 3.5%, and 4.0% (Excel file name was a typo; 4.5% was not included in this experiment).

Overall, 3.5% DMSO concentration seemed to be promising as it allows significant reduction of the false-positive signal while still allowing the positive signal to reach its maxima within the observed time period.

PlateMap_3 to 4.5.xlsx

DMSO 3 to 4.5 - Quantification Amplification Results.xlsx

DMSO 3 to 4.5 - Melt Curve Derivative Results.xlsx

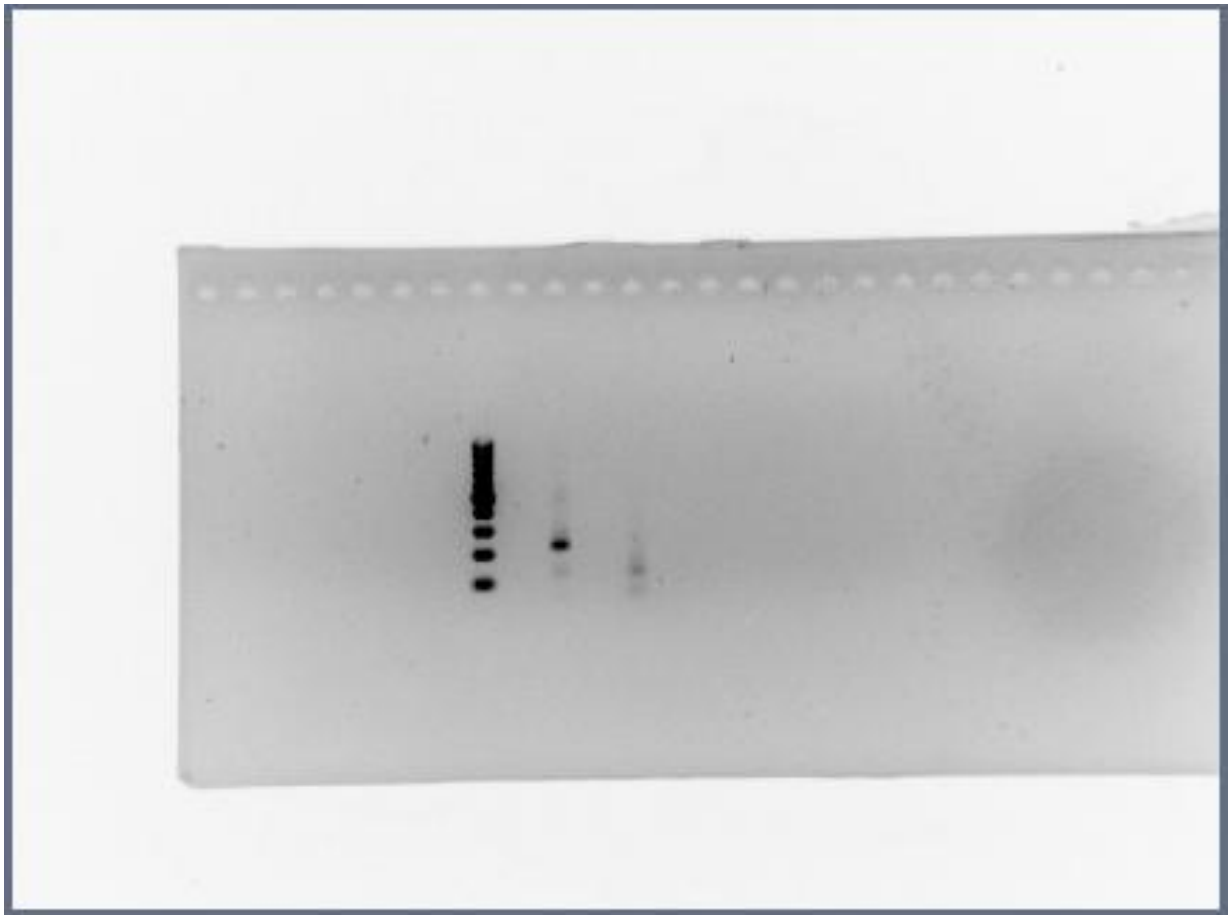
RPA Amplicon Confirmation with 2.5% DMSO

Project: Lab notebook
Authors: Sebastian Tommi Tandar
Created at: 2020-08-21T20:06:49.284461+00:00

FRIDAY, 21/8/2020

RPA was performed with or without the target template at 2.5% DMSO concentration. The reaction was performed at 50 uL scale for 20 minutes. The reaction product was purified using QIAGEN PCR CleanUp kit and ran on 2% agarose gel. 100 bp DNA ladder was used as marker. From left to right: 100 bp ladder, Template (+), Template (-).

RPA_2.5%DMSO.jpg



The correct band was formed on Template (+) lane (approx. 219 bp). A shorter smear was formed at Template (-) lane; indicating unspecific primer amplification. DMSO concentration might not be high enough to eliminate this unspecific amplification. Nevertheless, the formation of the correct amplicon was favored in the presence of a template.

Gel Doc primer screening (too low template concentrations)

Project: Lab notebook
Authors: Tom Langelaar
Created at: 2020-08-25T14:47:33.198368+00:00

TUESDAY, 25/8/2020

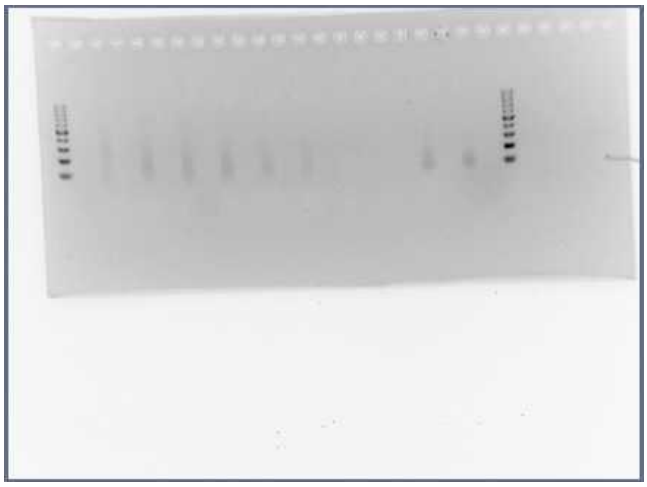
Now, RPA reactions were conducted with different templates: IA, SM, MB and PF.

However, due to an earlier mistake, templates were wrongfully diluted in series of 4x 1/100 instead of 1/10. Visible bands appear to be primers.

Table1

	A	B	C	D	E	F	G	H	I	J	K
1	Ladder	IA (+)	IA (-)	SM (+)	SM (-)	MB (+)	MB (-)	Negative contr	Negative contr	PF (+)	PF (-)
2											
3											
4											

screening templates 2.jpg



qPCR screening IA1 MB1 BS2 PF1 SC1 (primers and RPA sensitivity)

Project: Lab notebook
Authors: Tom Langelaar
Created at: 2020-08-25T14:41:29.932243+00:00

TUESDAY, 25/8/2020

Method

A screening of the primers above was carried out according to the protocol and 3,5% DMSO. The influenza, sacromycies and PF1 primers showed good results. Graphs can be found in the excel files.

-

The measurements shown in row A1-6 and B1-3 of the excel file (see table below for map) have been made to test the lower limit of RPA using different concentrations/copy numbers of yeast template for each reaction, while also including a negative control without template.

Table1

	A	B	C	D	E	F
1	A1: 4.23*10^6 copies	A2: 4.23*10^5 copies	A3: 4.23*10^4 copies	A4: 4.23*10^3 copies	A5: 4.23*10^2 copies	A6: 4.23*10^1 copies
2	B1: 4.23*10^0 copies	B2: 4.23*10^-1 copies	B3: 0 copies (negative control)			

lower limit and primer screening 25 AUG - Melt Curve Derivative Results.xlsx

lower limit and primer screening 25 AUG - Quantification Amplification Results.xlsx

Multiplex with SC/IA

Project: Lab notebook
Authors: Tom Langelaar
Created at: 2020-08-26T14:36:23.459559+00:00

WEDNESDAY, 26/8/2020

We have seen in previous experiments that the SC/IA primers seem to work, and that RPA is an effective way to amplify DNA strands. In this experiment, we will therefor investigate the cross-contamination of samples with either SC or IA in different ratios and see whether RPA is still a sufficient and reliable method for DNA amplifcation of the target.

The goal of this experiment is to find out whether amplification still happens when other DNA is present in the sample. We hope to expect that in especially favorable ratios with little contamination amplification can still happen and does only amplify the target gene.

Method
Kelly and Amber have tested both SC and IA as a mix with either one of primer pairs.

In the table below the volumes and ratios used can be found. However, due to larger ratios, some samples did contain a larger amount of fluid. (e.g. 5,5ul as opposed to 2ul)
We added 3 controls:
1 positive control containing only the target template
1 negative control containing no target template, and only "contaminated" DNA
1 negative control containing no DNA template at all

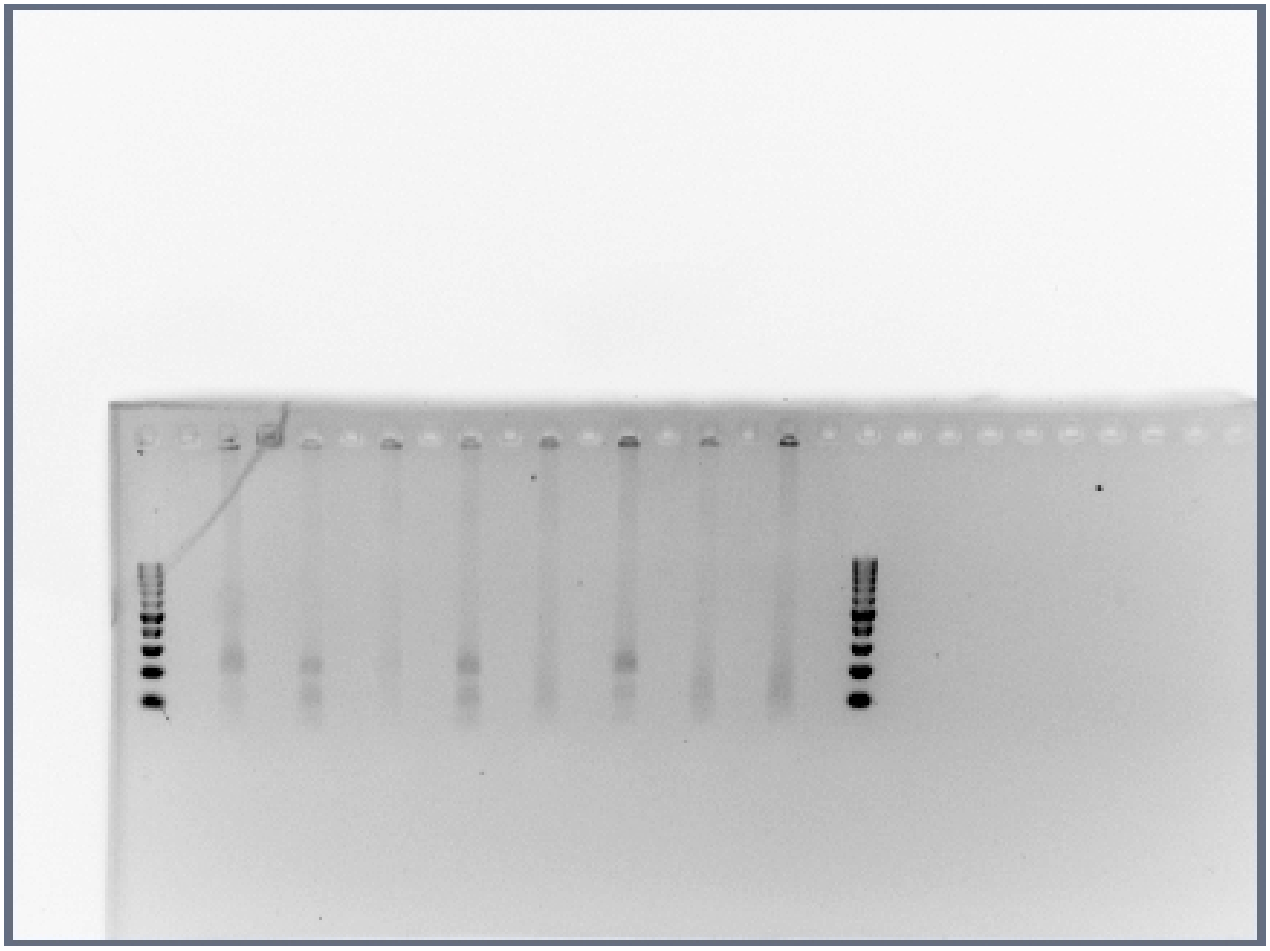
The positive control shows amplification happens for our target template, the first negative control shows specific amplification happens and does not amplify other DNA, and the last negative shows no amplification happens when no template is present.
For SC primers:

Table1

	A	B	C	D	E
1	Sample number	SC temp / IA temp	SC template (uL)	IA temp (uL)	
2	1	1:1	1	1	
3	2	1:2	1	2	
4	3	2:1	2	1	
5	4	1:10	0.5	5	
6	5	10:1	5	0.5	<-- possible pipetting error
7	6	1:0	2	0	
8	7	0:1	0	2	
9	8	Neg control	0	0	

SC primers:
When SC primers were used and run in the gel figure 1 shows us the results. Both negative controls showed no amplification, meaning the SC primers were specific enough for the template. The positive control shows as expected a band, meaning that amplifcation was succesful. Samples 1, 2, 4 and 6 also show bands indicating amplifcation, however 3 and 5 lack any bands. This is a peculiar observation as we expect 3 (2:1) and 5(10:1) to be more effective in amplification due to a higher amount of target template and less contamination as opposed to sample 2 (1:2) and 4(1:10). For unknown reasons a larger ratio of target DNA with little contamination resulted in no band indicating no amplification has happened. The other samples did show an amplification, even when SC template was present in a lower ratio. (1:10 and 1:2)

image.png



For IA primers:

While for the samples with the IA primers showed two negative controls and a positive control as expected, the actual samples show almost no clear amplification. Only the 4th sample shows a clear band aside from the positive control. This is unexpected, however, due to the higher ratio of "contaminated" DNA was present.

Table2

	A	B	C	D	E
1	Sample name	IA temp / SC temp	IA template (uL)	SC temp (uL)	
2	1	1:1	1	1	
3	2	1:2	1	2	
4	3	2:1	2	1	
5	4	1:10	0.5	5	
6	5	10:1	5	0.5	
7	6	1:0	2	0	
8	7	0:1	0	2	
9	8	Neg control	0	0	

image.png

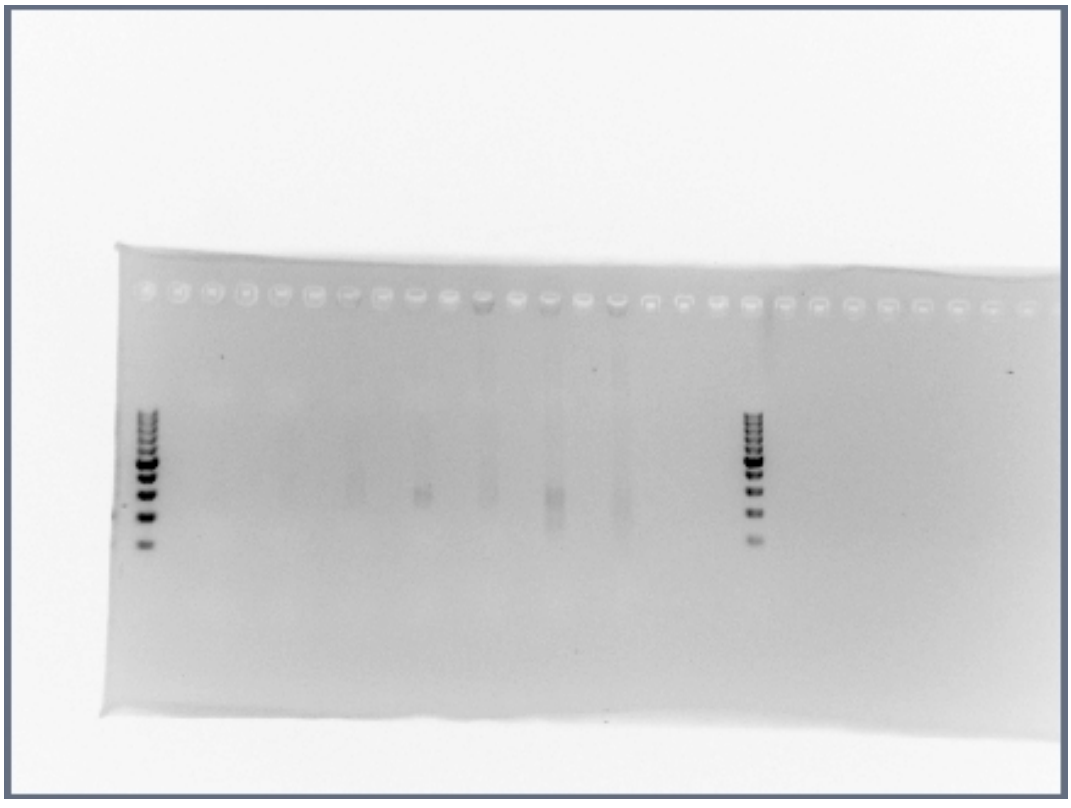
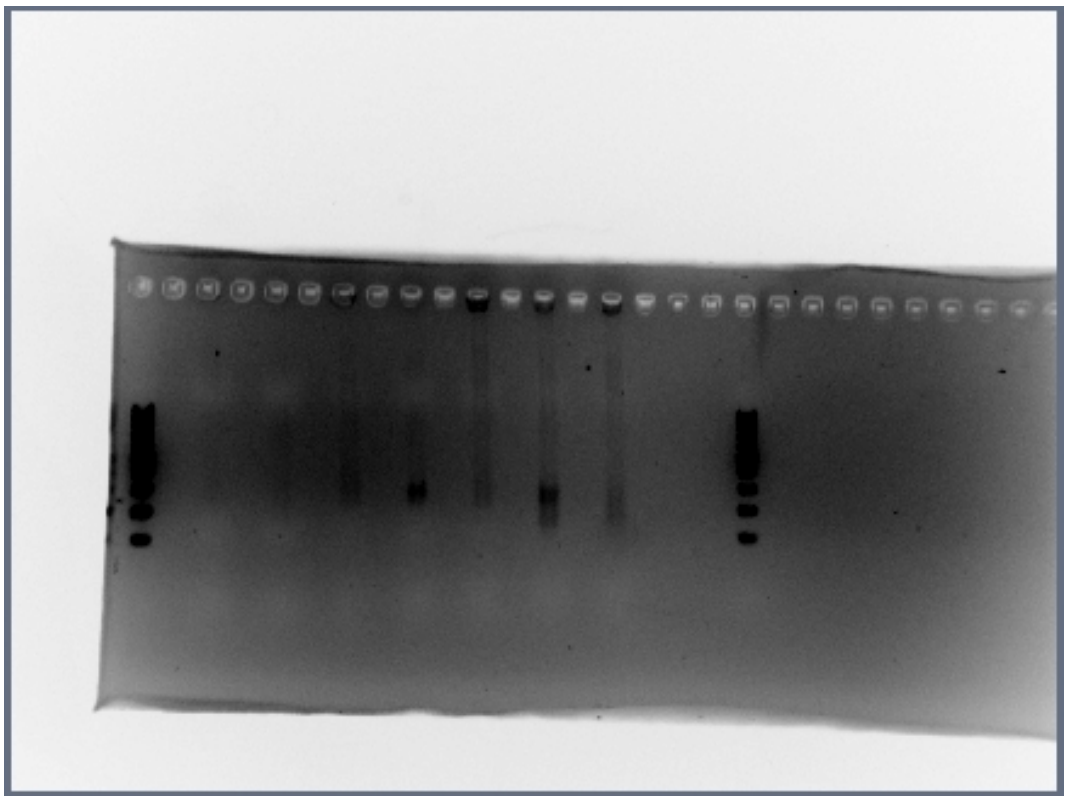


image.png



Conclusion

Whilst the controls worked generally properly in this experiment and where either negative or positive as expected, we did not always see amplification in the samples when expected and did see amplification when least expected in the lower ratios template DNA. Due to these inconclusive results we decided to redo this experiment.

Influenza A, different RPA incubation times

Project: Lab notebook
Authors: Tim van den Akker
Created at: 2020-08-28T08:21:39.406194+00:00

THURSDAY, 27/8/2020

Method

- Followed the modified protocol created by Sebastian, which includes the addition of DMSO and several other modification compared the original protocols (see 24-8-2020 for modified protocol). The protocol combines all methods (RPA, SDA and GQ) into one.
- Influenza RPA incubation was done for 10 and 20 minutes in duplicate to see whether the resulting unpurified RPA sample would yield enough construct for the following SDA reaction.
    - The 10 min. RPA incubation samples (D9-12) and their accompanying negative controls (H1+2 and H8+9) are shown in the table with a graph to the right for each of the wavelengths at which the absorbance was measured.
    - The 20 min. RPA incubation samples (D1-4) and their accompanying negative controls (D5-8) are shown in the table with a graph to the right.
    - The other columns are for another experiment.

Results

- In contrast to the RPA mix that was used for the proof-of-concept (with yeast), the DMSO volume for the Influenza samples was changed from 1.75µL to 1.5µL

iGEM_GQ_Oxidation TMB (Modified)_20200827_165707_tim.xlsx

iGEM_GQ_Oxidation TMB STOP SOLUTION (Modified)_20200827_170050_tim.xlsx

Multiplex with SC/IA redo

Project: Lab notebook
Authors: Tim van den Akker
Created at: 2020-08-31T08:31:01.124205+00:00

MONDAY, 31/8/2020

Due to the inconclusive results from the previous experiment we decided to redo this experiment.

Method

Amber has again tested both SC and IA as a mix with either one of primer pairs.

For SC primers (+results):

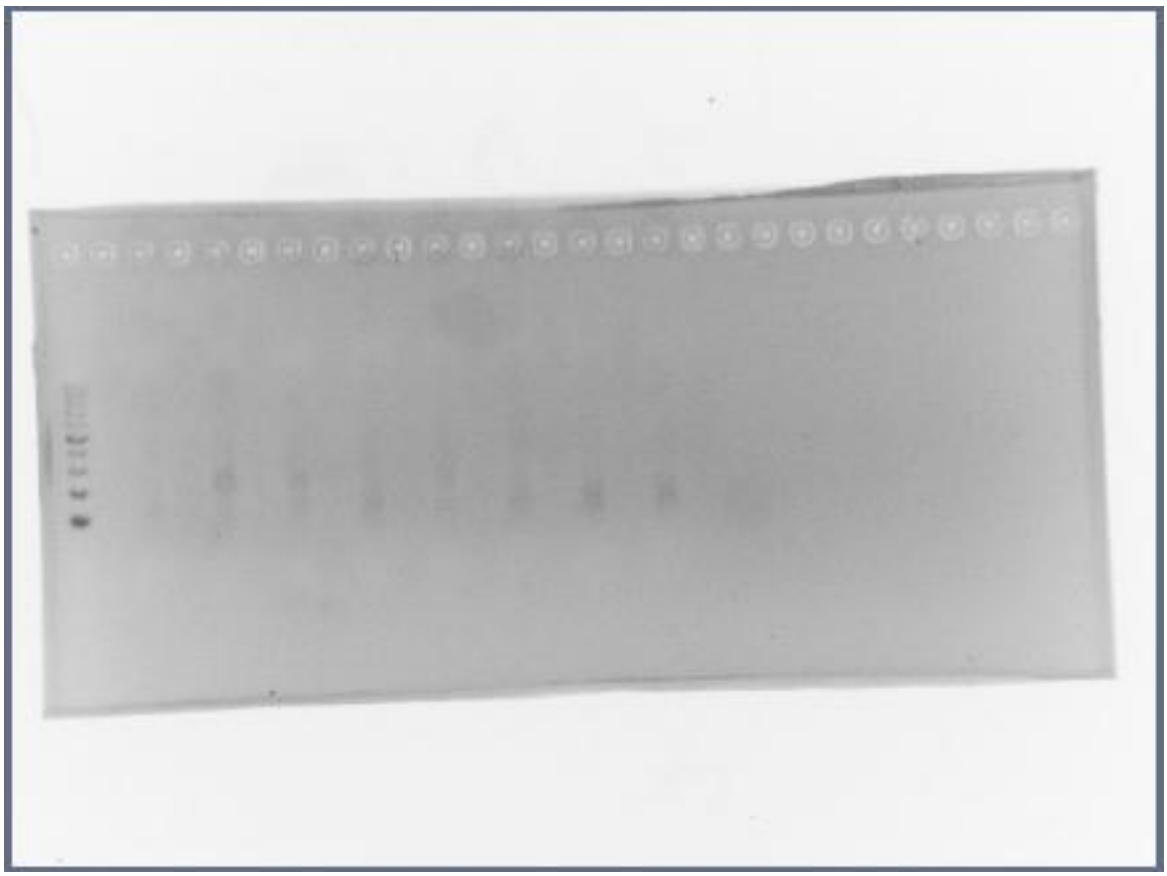
Table1

	A	B	C	D	E
1	Sample Number	SC temp / IA temp	SC template (uL)	IA temp (uL)	
2	1	1:1	1	1	
3	2	1:2	1	2	
4	3	2:1	2	1	
5	4	1:10	0.5	5	
6	5	10:1	5	0.5	<-- possible pipetting error
7	6	1:0	2	0	
8	7	0:1	0	2	
9	8	Neg control	0	0	

SC primers:

Only very faint lines are visible in the gel, unfortunately. Certain samples, 2, 3, 5 might show and indication of a slight band, but we cannot say for sure. In addition, the controls do not seem to have worked properly. The negative controls show a clearer indication of a band than the positive control.

SC multiplex.jpg



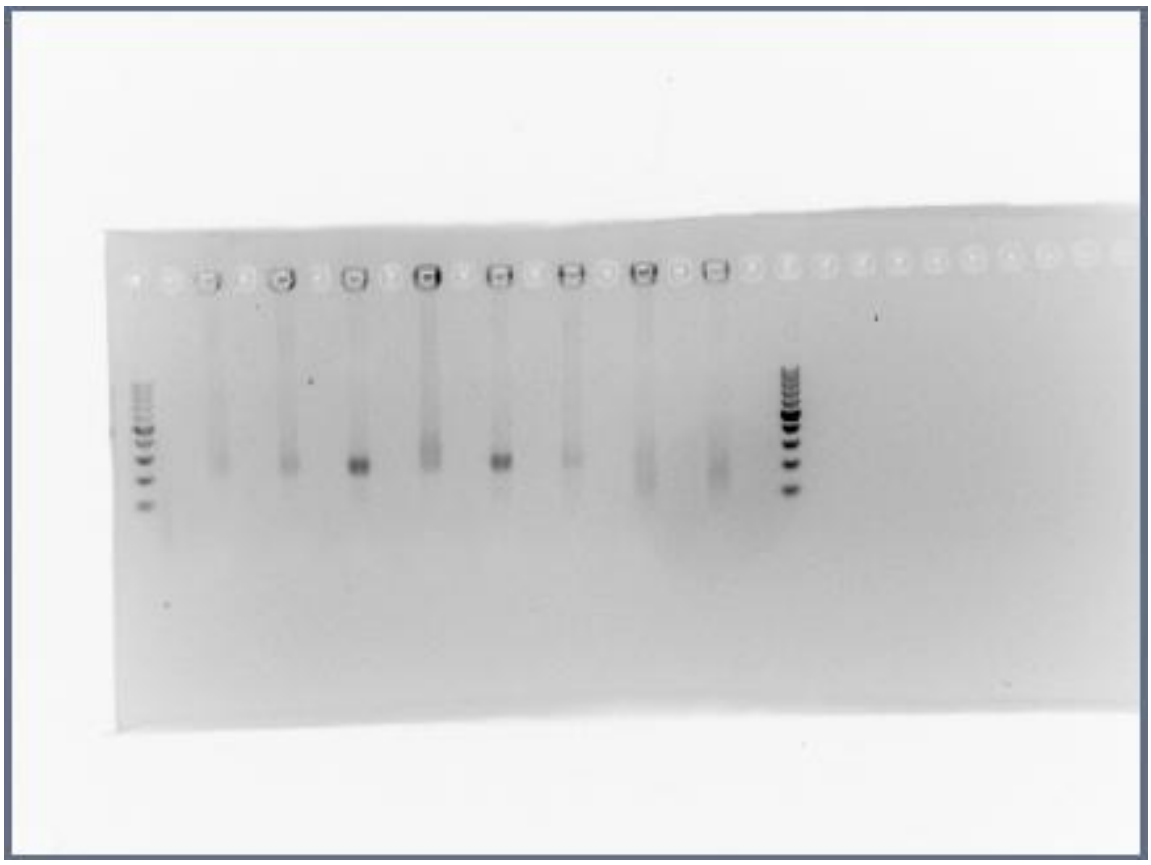
For IA primers (+results):

Table2

	A	B	C	D	E
1	Sample Number	IA temp / SC temp	IA template (uL)	SC temp (uL)	
2	1	1:1	1	1	
3	2	1:2	1	2	
4	3	2:1	2	1	
5	4	1:10	0.5	5	
6	5	10:1	5	0.5	
7	6	1:0	2	0	
8	7	0:1	0	2	
9	8	Neg control	0	0	

The positive control shows an expected band indicating amplification, while the negative controls don't. In addition, in all samples we see the expected amplification. However, in sample 3 and 5 we see a thicker band, This might be the result of the higher ratio of the these samples. However, this does not completely explain why sample 3 is not as thick as the control sample.

IA multiplex.jpg



Conclusion

Based on this experiment, and the previous yesterday, we have a slight indication that cross-contamination still allows some amplification to happen, even when smaller amounts of template DNA are present. However, not all samples, due to whatever reason, did not show signs of amplifcation. Where this would be due to pipeting errors, or a problem with the reaction would require more testing.