Validation protocol for WELPCR

Aim: To validate WELPCR

Objectives:

To isolate and quantify RNA

To synthesize cDNA, quantify and prepare 10-fold serial dilutions (107-102 copies)

To amplify cDNA and make endpoint analysis comparing WELPCR and commercial thermal cycler

To determine DNA concentration real-time by optical detection using Methylene Blue (MB) and compare with fluorescent detection using SYBR Green kit.

Protocols

1. Equipment specifications: Thermal cyclers—Electrophoresis equipment
2. Samples and sample preparation: Strain of interest—DNA extraction and purity checking—Different concentrations prepared by serial dilution of stock DNA
3. Amplification mix: Concentration—Recommended protocol
4. Primers: Concentration—Sequence (FP/RP)—Annealing temperature—Extension time
5. Amplification protocol: Identical PCR program and conditions for thermal cyclers under study
6. Documentation of PCR products: Visualization of PCR products by 1% AGE
7. Quantification: Total amount and purity of the amplified product can be determined by UV Spectrophotometer (using purified PCR product)
8. Determination of detection threshold at the lowest concentration of template DNA (Sensitivity)
9. Robustness: Validate reproducibility by same DNA concentration and target on thermal cyclers—AGE—Gel purification/ UV Spectrophotometer.

Reference:

González-González, E., Mendoza-Ramos, J. L., Pedroza, S. C., Cuellar-Monterrubio, A. A., Márquez-Ipiña, A. R., Lira-Serhan, D., ... & Alvarez, M. M. (2019). Validation of use of the miniPCR thermocycler for Ebola and Zika virus detection. *PLoS One*, *14*(5), e0215642.

Park, C. Y., Kim, J. D., Kim, Y. S., Song, H. J., Kim, J. M., & Kim, J. (2012). Cost reduction of PCR thermal cycler. *International Journal of Multimedia and Ubiquitous Engineering*, *7*(2), 389-394.

10. Real-time detection:
    a. Optical detection: Using 50μM Methylene blue (MB) and different DNA concentrations.
    b. Fluorescence detection: Using SYBR green dye for comparing EC results.
    c. Determine PCR efficiency and determine detection limit.

Reference:

Tseng, H. Y., Adamik, V., Parsons, J., Lan, S. S., Malfesi, S., Lum, J., ... & Gray, B. (2014). Development of an electrochemical biosensor array for quantitative polymerase chain reaction utilizing three-metal printed circuit board technology. *Sensors and Actuators B: Chemical*, *204*, 459-466

Wang, K., Chen, Y. P., Lei, Y., Zhong, G. X., Liu, A. L., Zheng, Y. J., ... & Chen, Y. Z. (2013). Electrochemical method for monitoring the progress of polymerase chain reactions using Methylene blue as an indicator. *Microchimica Acta*, *180*(9), 871-878.

Won, B. Y., Shin, S., Baek, S., Jung, Y. L., Li, T., Shin, S. C., ... & Park, H. G. (2011). Investigation of the signaling mechanism and verification of the performance of an electrochemical real-time PCR system based on the interaction of methylene blue with DNA. *Analyst*, *136*(8), 1573-1579.

Strain: Bacteriophage phi 6

1. Isolation of RNA from free viral particles

Kit: GenElute™ Total RNA Purification Kit

1.1. Protocol:

1. **Preparation of lysate:**

a. Transfer up to 100µL of viral suspension to an RNase-free microcentrifuge tube.

b. Add 350µL of Buffer RL. Vortex for 15secs in order to lyse the viral cells.

c. To this add 200µL of 96–100% ethanol. Mix by vortexing for 10secs.

2. **Binding RNA to column:**

a. Assemble the column with collection tube.

b. Add 600µL of lysate to the column and centrifuge for 1 minute at ≥3,500 × g (~6,000 rpm).

c. Ensure the entire lysate volume has been transferred. If required spin for an additional 1 minute at 14,000 × g (~14,000 rpm).

d. Discard the flowthrough and reassemble the spin column with its collection tube.

e. Repeat the above steps depending on the lysate volume.

3. **Column wash:**

a. Add 400µL of Wash Solution A to the column and centrifuge for 1 minute at ≥3,500 × g (~6,000 rpm).

b. Inspect the column to ensure the entire solution has passed through. If necessary, give an additional spin for 1 minute at 14,000 × g (~14,000 rpm).

c. Discard the flowthrough and reassemble the spin column with its collection tube.

d. Repeat above column wash steps for additional two times.
e. After final wash spin the column for 2 minutes to dry the resin completely.
f. Discard the collection tube.

4. RNA elution:

a. Place the column in a 1.7ml elution tube.
b. Add 50µL of Elution Solution A to the column.
c. Centrifuge for 2 minutes at 200 × g (~2,000 rpm), followed by 1 minute at 14,000 × g (~14,000 rpm).
d. Note the volume eluted from the column. If the entire 50µL has not been eluted spin the column for an additional 1 minute at 14,000 × g (~14,000 rpm).
e. For maximum RNA recovery, it is recommended that a second elution be performed into a separate microcentrifuge tube (Repeat the above steps).

5. Storage of RNA:

a. Purified RNA samples may be stored at -20 °C. For long term storage samples may be placed at -70 °C

2. RNA qualitative and quantitative analysis

RNA Quantification (Thermo Scientific Multiskan GO)

A ratio of 2.0 from A260:A280 indicates pure RNA.

2.1. Protocol

a. Select 'DNA/RNA' option under the cuvette tab on the home screen.
b. Select RNA from the menu.
c. Pathlength correction: Yes. Pathlength: 10mm
d. Clean the cuvette with 50µL of Milli Q water twice.

e. Set blank by using 50µL of MilliQ. Press the F2 key for Zeroing the instrument.
f. Clean the cuvette and add 49µL of Milli Q and 1µL of RNA sample to be quantified.
g. Insert the cuvette into the port and press the Start/On key to initiate the measurement.
h. Take each reading twice and note down the values.
i. After each reading clean the cuvette with Milli Q thoroughly.
j. When using different cuvettes, the blank has to be set up for each tube.

3. cDNA synthesis

Kit: iScript cDNA synthesis kit

Components	Volume per reaction (µl)
5x iScript Reaction Mix	4
iScript Reverse Transcriptase	1
Nuclease Free Water	14
RNA template (100fg-1µg)*	1
Total volume	20

*When using large amount of input RNA(>1µg), the reaction should be scaled up. Example: 40µl reaction for 2µg, 100µl reaction for 5µg.

Reaction Protocol:

Steps	Temperature	Time (minutes)
Priming	25°C	5
Reverse transcription	46°C	20
RT inactivation	95°C	1
Optional step	4°C	

4. Polymerase Chain Reaction

4.1. Reagents:

a. Primers: Sigma (503bp)

Oligo Name	Sequence
503bp Forward	5'-GAACCATATGACTTTGTACCTGGTCC-3'
503bp Reverse	5'-CAACGAATTCTCAGGCGCTTACCTCATC-3'

b. Master Mix: PCR Master Mix 2X (Cat. No. K0171) Thermo scientific
c. Nuclease Free Water
d. Template (cDNA)
e. Reaction volume: 20µL

Table 3: PCR reaction mixture

Reagents	**Volume per reaction**
Template (cDNA)	1µL
Forward Primer	1µL
Reverse Primer	1µL
Master Mix (2X)	10µL
NFW	7µL
Total Volume	20µL

3.2. Preparation of master mix:

a. In a sterile vial, add nuclease free water followed by Master Mix (2X).
b. Add forward and reverse primers and mix well.
c. Aliquot 19µL of master mix into each sterile PCR vials.

d. Add 1μL of isolated DNA to the master mix and ensure proper mixing of the contents.
e. Place the PCR tubes in thermal cycler with the program set to appropriate temperature and time.
f. After the PCR run is complete, PCR products can be visualized by Agarose Gel Electrophoresis or can be stored for further analysis.

Table 4: PCR thermal cycling conditions

Steps	**No. of cycles**	**Temperature**	**Time**
Initial Denaturation	1	95°C	120 sec
Denaturation	35 cycles	95°C	30sec
Annealing		52.8°C	30 sec
Extension		72°C	30sec
Final Extension	1	72°C	600 sec

*Annealing temperature depends upon primers

**Extension time will depend upon amplicon length

5. Quantification of amplified product

5.1. Agarose gel electrophoresis

1. Preparation of gel:

a. Gel tray was placed in casting apparatus and the combs were inserted into the place.

b. For preparation of 1% agarose gel, 0.5gm of agarose was weighed and dissolved in 50mL of 1X TBE buffer (Stock 5X).

c. The mixture was heated to dissolve agarose in the solution. Swirl the flask intermittently to check if agarose is dissolved completely.

d. Add 2µL of EtBr after cooling the gel for a few minutes.

e. The mixture was then poured into the prepared casting tray ensuring no air bubbles and allowed to set.

2. Preparation of tank buffer:

a. 5X TBE was diluted to 1X and 250mL (300mL) was used as tank buffer.

3. After the solidification of gel, the gel tray was placed inside the tank buffer and then the combs were removed.

4. 2µL of DNA sample (PCR product) was mixed with 1µL of loading dye and 7µL 1X TBE buffer and loaded into each well.

5. 0.5µL of DNA ladder mixed with 1µL of 10X FastDigest Green Buffer and 8.5µL of 1X TBE was added.

6. Voltage of 100V was applied and electrophoresis was allowed to run for 45minutes.

7. The gel was then visualised under a UV transilluminator.

5.2. Notes:

1. Loading dye: FastDigest Green Buffer (10X) Thermo Scientific
2. DNA Ladder: GeneRuler 1 kb DNA Ladder (Thermo Scientific)
3. Use a separate glove while handling the flask and EtBr vials to avoid direct contact with EtBr.
4. For eluting DNA from the gel (gel purified product) total volume should be between 30-40µL. Use loading dye accordingly (E.g. for 40µL use 4µL dye).
5. For only visualising the DNA band at ladder size

   2µL of DNA + 1µL 10X FastDigest Green Buffer + 7µL 1X TBE buffer

   0.5µL of ladder + 1µL 10X FastDigest Green Buffer + 8.5µL 1X TBE buffer

Table 6: TBE Buffer (5X) for 1litre	
Tris base	54g
Boric acid	27.5g
0.5M EDTA (pH 8.0)	20ml
pH	8.3

4.3. DNA extraction (Gel Purification)

a. Weigh a 1.5ml tube and note down the weight.
b. Excise the gel containing DNA band with a clean scalpel or razor and place it in a 1.5ml tube. Cut as close to the DNA as possible to reduce gel volume.
c. Record the weight of the tube with gel.
d. Add 1:1 volume of binding buffer to the gel slice. For e.g. 100µL for 100mg agarose gel.
e. Incubate the gel mixture at 50-60°C for 10minutes or until the gel slice dissolves completely. Vortex the gel mixture briefly before loading it onto the column.
f. Check the colour of the solution. The yellow colour indicates optimal pH for DNA binding. If the colour of the solution is orange/violet add 10µL of 3M sodium acetate (pH 5.2).
g. Transfer up to 800µL of solubilised gel solution to the purification column.
h. Centrifuge at 12,500 rpm for 1minute. Discard the flow through and place the column back into the same tube.
i. Add 700µL of wash buffer to the purification column.

j. Centrifuge at 12,500 rpm for 1minute. Discard the flow through and place the column back into the same collection tube.
k. Centrifuge the empty purification column for an additional 1minute at 12,500rpm to remove any residual wash buffer.
l. Transfer the purification column to another 1.5ml microcentrifuge tube.
m. Add 30μL of nuclease-free water to the centre of the purification column and incubate at 37°C for 2minutes. (Protocol: 50μL of elution buffer to the centre of the purification column and centrifuge at 12,500rpm for 1 minute).
n. Centrifuge at 12,500 rpm for 2 minutes.
o. Discard the purification column and store the DNA at -20°C
p. Perform qualitative and quantitative analysis of DNA as mentioned in 2.1 using appropriate parameters.

For real-time detection, 50μM MB is to be added to the PCR mixture.
(Reference: Tseng, H. Y., Adamik, V., Parsons, J., Lan, S. S., Malfesi, S., Lum, J., ... & Gray, B. (2014). Development of an electrochemical biosensor array for quantitative polymerase chain reaction utilizing three-metal printed circuit board technology. *Sensors and Actuators B: Chemical*, *204*, 459-466).

Validation Parameters

End-point analysis (*Guidelines for the Validation of Analytical Methods for Nucleic Acid Sequence-Based Analysis of Food, Feed, Cosmetics and Veterinary Products*, n.d.):

a. Sensitivity (LOD): Perform serial dilutions for target DNA and 12 replicates for each dilution level. Dilution with lowest copy number showing positive result for all 12 replicates is the approximate value of LOD. It can be expressed as concentration of DNA (ng or pg) or target copy number/reaction. Formula LOD = 3.3σ /S (where σ is standard deviation and S is the slope of calibration curve)

b. False negative (FN)/False positive (FP): FN occurs if the test result if negative but actual condition is positive (conc ≥ LOD); FP occurs when test result is positive but actual condition is negative. FN and FP rates determined should be <5%.

c. Robustness: It is the ability to remain unaffected by small deliberate deviations from experimental conditions.

Table 2. Example Robustness Testing Matrix* (n≥3)

Factor	*Combination*							
	1	2	3	4	5	6	7	8
PCR Equipment	A	A	A	A	B	B	B	B
PCR kit or reagent provider	X	X	Y	Y	X	X	Y	Y
Primer concentration	NC	-30%	NC	+30%	NC	-30%	NC	+30%
Probe concentration	NC	-30%	+30%	NC	-30%	NC	NC	+30%
MM Volume	-5%	-5%	+5%	+5%	+5%	+5%	-5%	-5%
Annealing Temp.	+1°C	-1°C	+1°C	-1°C	-1°C	+1°C	-1°C	+1°C

NC-No change from optimized conditions
MM-Master mix

**From: Guidelines for the single-laboratory validation of qualitative real-time PCR methods-Bundesamt fur Verbraucherschutz und Lebensmittelsicherheit-March 2016*

Reproducibility: Same DNA concentration amplified in replicates for both thermal cyclers (Everardo Gonzalez-Gonzalez et.al)
PCR efficiency curve: Amplicon product concentration vs target DNA concentration. Calculate R of curve fit and compare old and new thermal cyclers.

Real-time analysis

Standard curves: Generation of standard curves is required for optimization of PCR results. Used for determination of PCR efficiency, dynamic range and LOQ.
PCR efficiency: Measure of how close the observed reaction is to statistical doubling. Standard curve of Ct vs log of sample concentration, no. of template copies or dilution factor with average slope between -3.1 to -3.6 with efficiency of 90%-110% generally acceptable. Slope (y) = mx + b (where y = CT value and x = log target amount)
PCR efficiency (E) = $(10^{-1/m} - 1) \times 100$, where E = efficiency and m = slope of the linear standard curve.
Dynamic range: Concentration range over which target nucleic acid is reliably detected. A well-designed and well-optimized real time PCR assay will have a linear range of 6-8 orders of magnitude. The R^2 value of the standard curves used to determine the dynamic range should be ≥ 0.98.

Limit of quantification (LOQ): Lowest amount of analyte in a sample that can be reliably quantified. LOQ = $10\sigma / S$ (where σ is standard deviation and S is the slope of calibration curve)

Repeatability and Reproducibility Standard Deviation: $RSD_{repeatability}$ is the relative standard deviation of results obtained with the same method, by the same analyst, in the same laboratory, with the same equipment, on the same samples

(repeatability conditions). Should be <25% over the whole dynamic range of the assay. The relative standard deviation between laboratories ($RSD_{reproducibility}$) should be less than 35%.