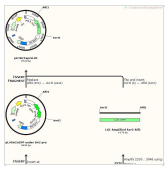

Apr 05, 2021

Development of a reporter line for assessing the changes in gene expression of *PfSir2a*

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Linda O Anagu: Special appreciation to Catherine J Merrick and Paul D. Horrocks

1 *Works for me* dx.doi.org/10.17504/protocols.io.btsxnnfn

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ABSTRACT

A luciferase reporter line of the malaria parasite, *Plasmodium falciparum*, was designed in an attempt to measure the activity of *PfSir2a* promoter independent of mRNA expression in response to stress. This can also be used to isolate any promoter activity-associated-stress response from in-parasite cellular feedback attempting to restore homeostasis due to an altered sirtuin mRNA level. Here, a luciferase reporter gene was cloned under the presumptive *Sir2a* promoter (~1.7kb of the gene's upstream sequence) and the reporter gene was transfected into 3D7 parasites. Its expression profile was similar to that of the endogenous *Sir2a* gene across the intraerythrocytic cycle. Luciferase reporters can be used to investigate the promoter activity of other genes.

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KEYWORDS

null, Luciferase reporter systems, epigenetics, sirtuins, promoter activity, Plasmodium falciparum

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GUIDELINES

Some materials are also mentioned in the tables when the steps were outlined.

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MATERIALS TEXT

- 1mm gap-width cuvette (Bio-Rad, UK)
- SOC broth

Components	Quantity
Tryptone	20 g
Yeast extract	5 g
NaCl	0.5 g
KCl	0.186 g
MgCl2	0.952 g
ddH20	Up to 1 L

This makes IL of SOC broth. The volume of ddH_20 was brought up to 900 ml and the pH adjusted to 7.4 with 10 M NaOH before finally making up the volume to 1 L with ddH_20. After sterilization of the media, 20 ml of sterile 1 M glucose was added immediately before use.

- LB (Luria-Bertani) broth (Fisher Scientific, UK)
- Qiaprep®Spin Miniprep Kit (QIAGEN GmbH, Germany)
- 5X TBE

A	B
Component	**Quantity**
Tris Base	54.00 g
Boric acid	27.50 g
EDTA	4.65 g
ddH2O	Up to 1L

This makes 1 L of 5X TBE. Adjust the pH to 8.3.

- AflII (also called BSPT1, (10 U/µL)) (Thermo Scientific, USA)
- AvrII (also called XmaJ1, (10 U/µL)) (Thermo Scientific, USA)
- Yellow buffer (Thermo Scientific, USA)
- QIAquick®Gel Extraction kit (QIAGEN GmbH, Germany)
- alkaline phosphatase (1 U/µL, Thermo scientific, USA)
- phenol/chloroform/isoamylalcohol (Thermo Scientific, USA)
- 3 M sodium acetate
- 1.5 ml microtubes
- yeast tRNA (Thermoscientific, USA)
- LB agar plate

Components	Quantity
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
ddH20	Up to 1 L

15 g of agar was added prior to sterilization to make LB agar.

- Qiaprep®Spin Miniprep Kit (QIAGEN GmbH, Germany)
- FastDigest enzymes (ThermoFisher, UK
- QIAGEN Plasmid Maxi Kit (QIAGEN GmbH, Germany)
- *P. falciparum* 3D7
- Incomplete CytoMix
- Red blood cells
- Blasticidin-S deaminase
- luciferase assay system (Promega, UK)
- 96-multiwell white plate (Grenier-bio-one, Germany)

BEFORE STARTING

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View the plasmid and purchase the enzyme needed.
Make or purchase competent cells.
Purchase all other materials.

Cloning of reporter luciferase gene under PfSir2A promoter

1 **Extraction and Digestion of the Vector Backbone** : The vector pLNSir2aGFP (or pLNSir2AGFP) with the *GFP* gene under *PfSir2A* promoter (reffered to as pLNSG vector here) and containing the AmpR gene

> Merrick CJ, Huttenhower C, Buckee C, Amambua-Ngwa A, Gomez-Escobar N, Walther M, Conway DJ, Duraisingh MT (2012). Epigenetic dysregulation of virulence gene expression in severe Plasmodium falciparum malaria.. The Journal of infectious diseases.
> https://doi.org/10.1093/infdis/jis239

was amplified by cloning this vector into competent *E. coli* (PMC103 strain, ATTC) via electroporation.

1.1 30m

The plasmid map of this vector (shown in the description section) was viewed in SnapGene software (GSL Biotech LLC) so as to identify the restriction enzymes needed to linearize the vector and remove the *GFP* gene.

1.2 35m

25 µl of gently thawed **On ice** competent cells was mixed with 1 µl of this plasmid. This mixture was left **On ice** ice for 15 min and then transferred into an ice cooled
1mm gap-width cuvette **Bio-rad Laboratories**

1.3 1h 15m

The cuvette content was electroporated (with a GenePulser II electroporator (Bio-Rad)) at 1.8 kV and the culture recovered in 300 µl of warm **37 °C** SOC broth for 1 h in a shaking incubator at **37 °C**

1.4 5m

The recovered culture was centrifuged at 6000 rpm for 1 min to form a pellet. 200 µl of the supernatant was discarded. The remaining supernatant was mixed with the pelleted culture.

1.5 10m

The mixture from 1.4 was added to 5 ml of LB (Luria-Bertani) broth (Fisher Scientific, UK) containing 5 µl of 100 mg/ml sterile ampicillin solution. This was incubated at **37 °C** overnight in a shaking incubator.

1.6 2h

The pLNSG plasmid from competent cells was extracted by using the Qiaprep®Spin Miniprep Kit (QIAGEN GmbH, Germany).

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1.7 The amount of extracted plasmid vector was determined using NanoDrop spectrophotometer 1000 (Thermo Scientific, USA). This was used to calculate the quantity of enzymes needed to linearize the vector.

1.8 3h

A large quantity of the extracted plasmid was digested in a 100 µl reaction, composed of the ingredients outlined in the table below, at **37 °C** for 2 h.

A	B
Component	Quantity (µl)
10X Conc. Yellow Buffer	20
pLNSG (46.3 ng/µl)	43.2
Enzymes	0.5 of each
ddH20	up to 100

The enzymes, AflII (also called BSPT1, (10 U/µL)) and AvrII (also called XmaJ1, (10 U/µL)) (Thermo Scientific, USA) was used to restrict the *PfSir2A* and *GFP* gene in pLNSG, making it linear. The most compatible buffer, yellow buffer (Thermo Scientific, USA), for both enzymes was selected. 1 unit of enzyme will digest 1 µg of DNA in 1 h at 37°C, so ¼ unit of enzyme was used based on the conditions and quantity of DNA being digested. Distilled/deionized water (ddH_20) was used.

1.9 The digested pLNSG was purified using agarose gel electrophoresis (0.8 percent agarose in 1X TBE, which was made from 5X TBE) and the level of purity/intactness assessed visually. 1h

1.10 The backbone (pLNSir2apro) was extracted from the gel after by using the QIAquick®Gel Extraction kit (QIAGEN GmbH, Germany). 1h

1.11 2h 15m

The extract from 1.10 was digested a second time using half of the original concentrations of enzyme and 0.5 µl of alkaline phosphatase (1 U/µL, Thermo scientific, USA) was added 30 min before the end of the digestion to reduce the chance of self-ligation.

1.12 15m

The double digested plasmid was then purified by phenol chloroform clean up and ethanol precipitation. Phenol chloroform clean up involved adding equal volume of phenol/chloroform/isoamylalcohol (Thermo Scientific, USA) solvent mixture and vortexing to extract all the enzymes and other contaminating protein/additives. This mixture was separated into an aqueous and non-aqueous layer by centrifugation at 8000 rpm for 1 min. The aqueous layer was collected using a pipette and put into fresh 1.5 ml eppendorf tube.

1.13 10m

For the ethanol precipitation, 0.1 volume of 3 M sodium acetate was then added to the aqueous layer and mixed. Thereafter, 2 volumes (2X volume of the above mixture) of absolute ethanol was added to the above, mixed and left at **-20 °C**, overnight or at **-80 °C**, for 2 h so as to de-salt and precipitate the DNA out from the solution.

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1.14 40m

Afterwards, the mixture was centrifuged at 4 °C , 14000 rpm, for 30 min. Ensure that the centrifuge is chilled before use.

1.15 20m

The supernatant was almost completely removed and the pellet formed was washed with ice cold 70% ethanol by spinning at 4°C, 14000 rpm, for 10 min.

2 **Purification and amplification of the *Fluc* insert** : The *Fluc* or simply *luc* (abbreviation for luciferase gene) coding sequence was amplified from the template pmPLP1, a generous gift from P. Horrocks laboratory, Keele University

Wong EH, Hasenkamp S, Horrocks P (2011). Analysis of the molecular mechanisms governing the stage-specific expression of a prototypical housekeeping gene during intraerythrocytic development of P. falciparum.. Journal of molecular biology. https://doi.org/10.1016/j.jmb.2011.02.043

using polymerase chain reaction (PCR) and the following *luc* primers; GATCCCTAGGATGCATGAAGACGCCAAAAA (LUCAvrII Forward) and GATCCTTAAGTTACAATTTGGACTTTCCGCC (LUCAflII Reverse).

2.1

The primers were designed using the SnapGene software and the oligonucleotide sequences were ordered from Eurofins mwg®. The primers had the AvrII and AflII enzyme site attached at the ends.

2.2 2h

PCR was carried in a reaction mixture containing the components are in the table below:

Component	Quantity (µl)
5X MyFi Buffer (Bioline, USA)	10
Template (1 ng/µl)	2.5
Primers (10 µM)	1 (0.5 each) (0.1 µM final concentration of each primer)
MyFi DNA Polymerase (Bioline, UK)	2
ddH2O	Up to 50

The PCR conditions were:

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Temperature (°C)	Time
95	1 min
95	15 s
55	15 s × 35 cycles
72	30 s
72	2 min

2.3 The PCR product insert was purified by agarose gel electrophoresis, extracted from the gel using the QIAquick®Gel Extraction kit (QIAGEN GmbH, Germany) and restriction digested with AflII and AvrII. 3h 20m

2.4 The digest was then purified by phenol chloroform clean up and ethanol precipitation. 1 µl of the cleaned up PCR product and plasmid vector backbone were analysed by using agarose gel electrophoresis and the NanoDrop spectrophotometer.

3 Ligation of the backbone and the insert, and detection of positive clones

3.1

The *luc* insert and linearized plasmid vector were ligated using a T4 ligase (Thermoscientific, USA) in a thermocycler at **16 °C** , overnight. The formula used for this reaction is shown in the table below. A control reaction was included to determine the extent of self-ligation or re-cloning of uncut vectors.

A	B	C
Component	**Test (µl)**	**Control (µl)**
10X ligase buffer (Thermo Scientific, USA)	2	2
Plasmid pLNSir2apro backbone (18.75 µg/ml)	0.8 (15 ng)	0.8 (15 ng)
T4 ligase (5 U/µl)	0.5 (2.5 units)	0.5 (2.5 units)
luc PCR product insert (18.75 µg/ml)	8 (150 ng)	-
ddH2O	Up to 20	Up to 20

Insert to vector molar ratio is 43:1. The final DNA concentration was ~8 µg/ml

3.2

The resulting pLNSir2aproluc (*PfSir2A-luc* reporter) construct was purified by phenol chloroform clean up and precipitated using the ethanol precipitation method **go to step #1.12** - **go to step #1.15** . 1 µl of yeast tRNA (Thermoscientific, USA) was added to a final concentration of 0.2 µg/µl, before the sodium acetate was added during the clean-up of the construct.

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3.3 30m

The construct was dissolved in 10 µl of ddH_20 and 5 µl of the construct solution was then used to transform 50 µl of electrocompetent *E. coli* by electroporation as described **go to step #1.2** - **go to step #1.3** . The recovered culture was centrifuged at 6000 rpm for 1 min to form a pellet.

3.4

200 µl of the resulting clear supernatant were discarded and the bacterial pellets were gently mixed with the remaining supernatant and cultured by spreading on an oven-dried sterile LB agar plate containing 100 µg/µl of ampicillin. This plate was incubated at **37 °C** , overnight.

3.5

The ratio of colonies on the test plate (*luc*: pLNSir2apro ligation) to the control plate (pLNSir2apro alone) was checked, then a number of colonies from the test plate was subcultured separately in 2 ml LB broth, containing 100 µg/µl of ampicillin at 37°C, overnight, in a shaking incubator.

3.6 4h

The plasmid was extracted from the resulting broth culture of each colony using the Qiaprep®Spin Miniprep Kit (QIAGEN GmbH, Germany) and digested using AvrII and AflII. The digest was run on the gel to identify positive clones.

3.7 4d

Plasmids from two positive clones were sent for sequencing by Eurofins mwg® using 4 sequencing primers; GATCCCTAGGATGCATGAAGACGCCAAAAA (LUCAvrII Forward), GATCCTTAAGTTACAATTTGGACTTTCCGCC (LUCAflII Reverse), GGGGTGATGATAAAATGAAAG (hsp86term_Seq) and GCAATTGTTCCAGGAACCAG (LUC_Seq) generated in Snapgene software (GSL Biotech LLC). The sequence trace was then aligned with the original sequence in Snapgene software (GSL Biotech LLC) and any mutation was verified.

4 **Complete restriction analysis of the plasmid construct** : *PfSir2a-luc* construct were restricted using 5 restriction enzymes that cleave at various restriction sites in the plasmid which resulted in different sized segments containing the different components of the plasmid.

4.1 2h

The following FastDigest enzymes (ThermoFisher, UK) were gently thawed and used to restrict the *PfSir2A-luc* construct: EcoRV, AvrII, ApaI, SacI and SalI. The reaction components and conditions are presented in the table below.

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A	B	C	D	E	F	G
Components/Conditions	**Reaction quantity/condition in each digest**					
	Tube 1	**Tube 2**	**Tube 3**	**Tube 4**	**Tube 5**	**Tube 6**
10X FastDigest Green Buffer (µl) (ThermoFisher, UK)	1	2	2	3	4	5
PfSir2A-luc construct (µl)	1	2	2	2	2	2
Number of enzymes	-	1	2	3	4	5
ddH20 (µl) up to	10	20	20	30	40	50
Incubation time (min)	20	5	10	13	20	20
Incubation temperature (°C)	37	37	37	37	37	37

Tube 1 is the control reaction. For other tubes, 1µl of each enzyme was added with the previous enzyme(s) being compulsorily part of the reaction of the reaction component. For example, in tube 3 a second enzyme was added in addition to the enzyme that was added to tube 2. The reaction was set up and the components put in the order described by the manufacturer.

5 **Preparation of maxi preps for large scale extraction of PfSir2A-luc reporter construct** : This protocol is similar to plasmid mini prep but involves the use of supersize kits and equipment needed for preparation of large amounts of plasmids used in transfecting large populations of protozoa or mammalian cells, and for low copy number plasmids.

5.1

E. coli carrying the *PfSir2a-luc* reporter construct was grown in 500 ml LB broth, overnight in a shaking incubator at 37°C.

5.2 The plasmid was then extracted from the resulting culture using the large scale QIAGEN Plasmid Maxi Kit (QIAGEN GmbH, Germany) with a yield of 578 µg. Digestion of a small aliquot of the extracted plasmid construct was used to validate the plasmid. 5h

5.3 1h

The plasmid was precipitated by ethanol precipitation and 50 µg was dissolved in 30 µl of ddH_20. This was then used for the subsequent transfection of *P. falciparum* 3D7.

Transfection of P. falciparum 3D7 with PfSir2A-luc reporter construct and characterization of the transgenic parasite line. 1w 6d 8h 10m

6 **Transfection of *P. falciparum* 3D7 with *PfSir2a-luc* reporter construct** : *P. falciparum* 3D7 was transfected with *PfSir2a-luc* reporter construct. This was performed as previously described

> Deitsch K, Driskill C, Wellems T (2001). Transformation of malaria parasites by the spontaneous uptake and expression of DNA from human erythrocytes.. Nucleic acids research.

with some modifications.

6.1 50 µg of the plasmid was first introduced into uninfected red blood cells (RBCs) that had been washed in incomplete CytoMix, 1h

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Editors Robert Ménard. Malaria: Methods and Protocols.
http://10.1007/978-1-62703-026-7

. This introduction was carried out by electroporation of a mixture of 30 µl of a solution of the plasmid construct (1.67 µg/µl) in sterile ddH_2O, 170 µl of CytoMix and 200 µl of the washed RBCs, in a 0.2 cm cuvette at 0.31 kV and at 960 or 950 µF (microfarads), aiming for a time constant of between 10 to 14 ms.

6.2  30m

The DNA loaded RBCs were then recovered with 5 ml of complete media and washed twice with the complete media in a Falcon tube.

6.3 30m 0.5 ml of the parasites in untransformed RBCs at about 5% parasitaemia was then cultured in 4.5 ml of the transformed RBCs in complete media at ~2.5% haematocrit.

6.4  2d

They were allowed to multiply, reinvade the transformed RBCs, and spontaneously take up the introduced plasmid. 2 µg/ml (of the final culture volume) of Blasticidin-S deaminase (BSD), a drug that selects for the transfected parasites, was then added 48 h later.

6.5 The parasite culture media was regularly changed in order to remove dead parasites and refresh the parasite nutrients. The drug was added with each media change until drug resistant parasites emerged and thereafter.

6.6 

50 µl of 100% RBCs was added every week until the transfected parasites came up. Parasite detection was done by microscopy and bioluminescence signal was measured.

7 **Detection of transgenic parasites** : The protocol used to detect this luciferase activity of parasite culture was the improved Single-Step Lysis Protocol 35m

Hasenkamp S, Wong EH, Horrocks P (2012). An improved single-step lysis protocol to measure luciferase bioluminescence in Plasmodium falciparum.. Malaria journal.
https://doi.org/10.1186/1475-2875-11-42

. This was done using a luciferase assay system (Promega, UK) and a luminometer, Glomax-Multi Detection System (Promega, UK). The luciferase assay system contained 5X Passive lysis buffer (PLB) and Luciferase assay substrate (LAS).

7.1 20m In triplicate well per sample, 10 µl of 5X PLB, warmed to 37°C, was homogenized with 40 µl of parasite culture in each of the wells of a 96-multiwell white plate (Grenier-bio-one, Germany), by rocking/swirling.

7.2 10m 50 µl of LAS was then added to the mixture in each well at room temperature and mixed by

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rocking/swirling.

7.3 The bioluminescence was measured in light unit using the Glomax-Multi detection immediately. 5m

8 Characterization of 3D7 *PfSir2A-luc* reporter line 1w

8.1 Asynchronous parasite population of 3D7 *PfSir2A-luc* reporter line was used to determine the effect of parasitaemia on bioluminescence by serial dilution from 2.5% parasitaemia down to 0.5% parasitaemia

> Constantly mix the parasite culture while diluting so as to ensure even distribution of the parasites.

.

8.2 Additionally, tightly synchronized parasites were used to determine the bioluminescence of the 3D7 *PfSir2A luciferase* reporter line, alongside the well characterized Dd2 *luciferase*, *Pfpcna-luc,* reporter line 2d

> Wong EH, Hasenkamp S, Horrocks P (2011). Analysis of the molecular mechanisms governing the stage-specific expression of a prototypical housekeeping gene during intraerythrocytic development of P. falciparum.. Journal of molecular biology. https://doi.org/10.1016/j.jmb.2011.02.043

throughout their IDC. The experiment was begun 55 h and 48 h after the Percoll and sorbitol synchronizations respectively (i.e. 7 h post invasion (hpi)) and the parasites conformed to the required stage as assessed by Giemsa stained thin blood smear which indicated at least 82% purity of the required stage.

8.3 The parasite samples were harvested when they were at most 7, 10, 21, 24, 27, 30, 45, 48, 51, and 54 hpi and their bioluminescence determined **go to step #7** . The signal was normalized to 1% parasitaemia. 2d

8.4 A thin blood smear was also made for the different time points, stained and photographed using Leica® camera attached to a light microscope. 5h

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