P. falciparum 96-well plate Transfection Protocols

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1. Reagent List and Materials Information

Cell Line	Supplier	Passage Number
Plasmodium falciparum 3D7	NIH	TBD
Plasmodium falciparum W2	ATCC MR4 (MRA-157)	TBD

Material	Catalog #	Supplier
96 well round bottom plates	Fisher	07-200-95
96 well v-bottom plates	Fisher	07-200-96
96 well flat bottom plates	Fisher	07-200-90
384 well flat bottom non-sterile plates	Corning	3573
Whole Blood Leukocyte Reduction Filter	Fenwal, Inc.	4C2486
MACS Separation Columns	Milteny Biotec	130-042-901
Cell Line 96-well Nucleofector Kit SE	Amaxa	VHCA-1001
GenElute HP Endotoxin-free Plasmid Maxiprep Kit	Sigma	NA0410
Renilla Luciferase Assay System	Promega	E2820
96 well custom slides: 127.80mm x 85.50mm x 1mm thick, ground edge, washed, white glass w/ 7mm Superfrosted tab	Thermo Fisher Scientific	DRS-2101P
PVDF 0.45um, long Drip	Thomson Instrument Company	982125

Reagent	Catalog #	Company
Gentamicin	Invitrogen	15750-078
Giemsa	Giemsa	10092-013
Hepes	Sigma	H3375-1KG
Hypoxanthine	Sigma	H9636-25G
RPMI	Invitrogen	313800-022
Sodium Bicarbonate	Sigma	56297-1KG
Sorbitol	Sigma	S3889-1KG
Albumax II	Invitrogen	11021-045
NaATP. Adenosine 5’-triphsophate disodium salt	Sigma	A3377
Recombinant R. reniformis Luciferase	RayBiotech	RB-15-0003P

Biomek Materials	Supplier	Catalog Number
12-channel trough	E & K Scientific Products	EK-2034-S
250uL tip boxes	VWR	82018-028
20uL tip boxes	USA Scientific	1061-2400

2. Transfection

2.1 Days before transfection

Blood
Fresh human blood should be drawn from a donor as close to the transfection date as possible, preferably, on transfection day.

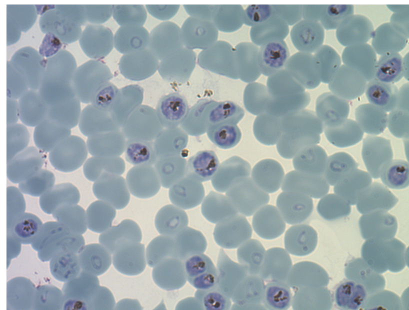
Culture

a. Synchronize parasite culture 3 days before transfection, taking into account that parasites have to be at around 5-10% parasitemia, 2% HC, in schizont stage the day of transfection.
b. 24 hours before transfection split the ring stage parasites to 1% HC.

Note: we have observed that faster growing strains tend to emerge from selection pressure in the following order: W2 > D10 > 3D7

2.2 Transfection Day

Culture
Make a smear to make sure that the culture is at around 5-10% healthy looking schizonts (at 1% HC). See picture below:



Blood
Resuspend around 1 ml of 100% RBCs in 49 ml of RPMI wash, spin at 1500rpm at room temperature for 8 min, no brake. Repeat 3 times.

Recovery plate preparation
Place 200μl of RPMI complete into each well of a 96-well, flat bottom, plate and place in the gas-controlled incubator.

Nucleofector 96-well Shuttle System Setup
Make and save a parameter file for the transfection you are about to perform. Controls include no pulse, no DNA. Make sure that the Nucleofector II, 96-well Shuttle, and PC are communicating. It helps to shut off the screen saver and upload the parameter file to the nucleofector ahead of time. The optimized pulse setting is CM-162.

Preparation of Transfection Mixture

a. Add the Supplement to the 96-well Nucleofector Solution SE at a proportion of 1:4.5 and mix by flicking the tube. You can also use the Cell & Reagent calculator on the Lonza website. Write down the date on the SE solution tube, now ready to use and stable for 3 months at 4°C.

b. Prepare the following mixture:

20 ul	Solution SE Solution (supplement added)
2 ul	Na_2ATP 200 mM in 50mM K^+phosphate pH 7.4
2.5 - 10 ug	plasmid DNA (4 μl max volume)
6 ul	RBCs 100% (washed 3 times)

Important: RBCs should be added last to the transfection mix, immediately before nucleofection. Work as quickly as possible because leaving RBCs in SE Solution for extended periods seems to cause lysis leading to reduced transfection efficiency and viability.

Note: If working with multiple plasmid constructs we recommend assembling a v-bottom 'DNA plate' where each construct is pre-aliquoted into the wells. Add Solution SE/Na_2ATP mixture to each well and last of all transfer RBCs using a multi-channel pipette.

c. Gently mix washed RBCs with the DNA/Solution SE/ATP mixture and transfer only 20 μl of the mixture to the electroporation plate (leftover transfection mixture will remain). Avoid air bubbles while pipetting. Inappropriate pipette tips can get stuck in the wells of the Nucleocuvette modules.
d. Cover the electroporation plate and tap the electroporation plate 2-3 times on the bench to get rid of bubbles.
e. Apply pulse as soon as possible.

Electroporation

a. Place the 96-well electroporation plate with lid into the retainer of the 96-well electroporator. Well "A1" must be in upper left position. Press "Upload and start" to apply pulses.
b. After complete program execution and retainer opening, carefully remove the 96-well electroporation plate from the retainer.

Culturing post-electroporation

a. Quickly place the electroporation plate in the incubator for 5 min to recover.
b. Rapidly but gently, transfer 80ul of the pre-warmed medium from the recovery plate to the electroporation plate from the pre-warmed plate and put the plate back into the incubator for 15 minutes.
c. Transfer the electroporated RBCs (eRBCs) and medium (~100ul) back to the pre-warmed plate.

d. Gently pellet the eRBCs for 2 min at 90xg (720 rpm, rcf=90g), remove 180ul of supernatant, then add 180ul of warm RPMIc
e. Incubate for 3-4 hours before adding late-stage MACS purified parasites.

MACS purification of Schizonts

a. Change the medium in the culture flask and bring it to 2%HC with fresh RPMIc.
b. Place the MACS column in the magnet, and add 5 ml of culture to the column reservoir.
c. Allow the culture to flow under gravity, and cover the column with the lid to a 50ml falcon tube.
d. Catch the flow through in a 50 ml falcon tube.
e. When the culture reaches the top of the magnetic beads on the column, add 5ml of RPMIc to the column to wash away unbound RBCs. trophozoites and schizonts should remain bound to the column. You are done when the flow through runs clear.
f. Add 5mls RPMIc to the top of the column. Remove the column from the magnet, and place it over a NEW 15ml Falcon tube. Insert the plunger into the column and gently push the liquid through the column.
g. Smear the eluate. It should be about 99% trophozoites and schizonts. Count by hemocytometer.

Note: MACS purification efficiency:
From 43 ml of a 8% parasitemia (R: 16, T: 47, S:9), 2%HC, culture the recovery is: 1.7 x 10^7 schizonts/ml (in 5ml, 8.5 x 10^7 schizonts). The efficiency of the MACS purification is 3%. With our homemade magnets, the efficiency is 5%. Therefore one 50 ml flask of a high parasitemia culture at 2%HC should be enough for a whole 96-well transient transfection plate.

Infection of eRBCs

Depending on the application, whether transient or stable add the following number of parasites to each well of eRBCs.

<u>Stable transfection:</u>
Add 5% (1x10^6) MACS purified schizonts to each 200 ul 1%HC transfection well.

<u>Transient transfection:</u>
Add 2% (4x10^5) MACS purified schizonts to each 200 ul 1%HC transfection well.

Note: Due to lysis of RBCs during electroporation, final HC is reduced from 3% to ~1% HC.

3. High-throughput Luciferase Assay

A detailed video of the entire protocol is to be found on youtube at: http://www.youtube.com/watch?v=gVzo_3gOWqQ

Before you begin:
- You will need a 96-well vacuum manifold and PVDF filters
- Prepare a fresh 0.003% saponin/1XPBS solution
- Let the Promega Renilla Luciferase kit components equilibrate to room temperature.

a. 48 hs after transfection (schizont stage parasites) remove all but 20ul of the culture supernatant media.
b. Place 0.45um PVDF-bottom filter plate onto the vacuum manifold. Tape off unused wells.
c. Add 200ul of 0.003% saponin/1XPBS to each well and incubate 90 sec. *Note: The cells will not lyse immediately, but lysis will be apparent when wells become translucent. If you don't wait for complete lysis, the filter will clog. We have empirically determined that using higher concentrations of saponin will also clog the filter.*
d. Transfer 100 of lysed culture onto the 0.45um PVDF-bottom filter plate, and apply vacuum.
   Note*: The filter capacity limit is 200 ul of a 14% parasitemia culture at 1.33% HC (1.86 x 10^7 parasites/ml or 3.72 x 10^6 parasites).*
e. Once the lysate has passed through, wash once by adding 200ul 1X PBS onto each well under vacuum.
f. Once wash has filtered through completely, close vacuum source and place the filter plate top of a u-bottom plate. Tap-spin the plate for 30sec at 514 xg to remove wash liquid drops.
g. Place the filter plate over the white-well luciferase assay plate, and add 30ul of 1X *Renilla* luciferase lysis buffer (in H_2O) to each well onto the filter surface.
h. Incubate 15min for lysis to proceed. During this time prepare 1X Renilla substrate in *Renilla* assay buffer (not water!).
i. Spin the plates (stacked) at 913 xg for 5 min to completely elute the lysate.
   Note: The lysate in the white-well assay plate should be clear (not red). Red on the surface of the PVDF filter means incomplete RBC lysis. A brown filter surface is normal, retained hemozoin.
j. Read Luciferase signal on a luminometer. We were most successful using the Veritas Microplate luminometer (Turner Biosystems). Inject 100ul of Promega Renilla Luciferase assay reagent at 1X per well. Settings: 2 sec delay, 10sec integration time. As a positive control use 1 ul of a 1:1 x 10^6 dilution of the recombinant *R. reniformis* Luciferase (stock: 1 ug/ul, 4 x 10^{10} RLU/ug)
   Note: Renilla Luciferase is stable in the assay lysis buffer for some period of time. So there is no need to work fast or in the cold.

4. Stable transfection

4.1 Positive selection

Day 0: Transfection day. Feed the parasites from now on every day once.
Day 1: Split ½ to ~0.5% HC. *We recommend keeping the split as a duplicate.*

Day 2: Add 1.5% HC to reach ~2%HC final. Make a smear and make sure culture is not higher than 5% parasitemia, if so, split back.
Add medium containing the appropriate positive selection drug (2.5 nM WR99210 or 500 ug/ml G418). From today on parasites are always under drug pressure until they come up.
Day 7 or 8: Add 2%HC to bring cultures up to 4%HC. Keep culturing at 4%HC from now on.
Day 11: Add 0.5%HC on top to replace lysed or old RBCs. Repeat this step once a week from now on.
Day 12: Smear the wells. Usually no parasites are observed at this stage. From now on smear wells twice a week.
Day 15: Replace 30% of the culture with fresh 4%HC media. This is done to replace lysed or old RBCs. Repeat this step once a week from now on.
Day 17: Get fresh blood from a donor.
Day 18: Split the entire culture plate ½ with the freshly obtained blood. Parasites start appearing from this day on.

Important Notes:

- *These cultures are VERY susceptible to growth conditions. Handle them with care and ALWAYS make sure the pH of the media is correct. Transfected cultures will NOT come up if pH of the media is slightly off.*
- *Always add the freshest blood possible.*
- *Dead parasites often can be confused with live schizonts. We consider a culture well alive and emerging, only when we see rings. Any other troph- or schizont-like forms are NOT considered as proof of a culture that is coming up.*
- *Once culture wells are up we recommend keeping wells continuously under positive selection and split some of the culture from these wells to put under negative selection.*

4.2 Negative selection

a. As soon as the culture under positive selection pressure reaches 1-5% parasitemia apply the corresponding negative selection pressure (appendix A). Parasitemia will decrease and 2 weeks later resistant parasites will arise.
b. Check for target locus disruption by whole cell PCR (see below).
c. Single cell clone knockout candidates.

5. *P. falciparum* whole cell PCR

a. Take 20 ul culture aliquots from wells at 0.1-10% parasitemia, 4% HC, quick spin to pellet and remove supernatants completely.
b. Add 40 ul ice-cold H_2O and quickly transfer 10 ul to PCR mix on ice.

PCR Mix:
10 ul lysed whole cells

1.2 ul H_2O
4 ul Phusion HF Buffer 5X
0.4 ul dNTPs 80%AT 10mM
2 ul Primer 1 5uM
2 ul Primer 2 5uM
0.4 ul Phusion polymerase (0.8 U final)
20 ul total volume

Cycling parameters:

	35 x		
98 ˚C	98 ˚C	60 ˚C	60 ˚C
2 min	10 sec	1min/kb	4min

6. Modified MSF Assay

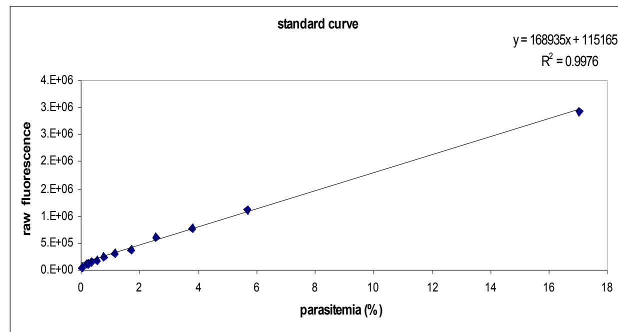
mMSF

The malaria SYBR green assay is performed according to Smilkstein et al. (26), with modifications.

a. Remove spent cell culture media from wells and replace with fresh media
b. Prepare a mixture of 2:1 MSF lysis buffer and PBS 1X with 1X SYBR Green I (Molecular Probes) and aliquot 30 ul of this mixture into each well of a Corning 384-well black assay plate.
c. Transfer 10 ul of the resuspended culture to the assay plate and mix.
d. Read plates on an Analyst HT (Molecular Devices) with 485 nm excitation, 530 nm emission and 505 nm dichroic, for 40 msec per well with a 1% neutral density filter to allow linear signal acquisition on the SmartRead 2 setting.

Make a standard curve

a. Count smear of culture with parasitemia >10%.
b. Transfer 10ml of highly parasitized cells to a sterile trough.
c. Prepare 25ml, 2% RBCs.
d. Transfer 100ul of 2% RBCs to columns 2:11.
e. Transfer 200ul of parasitized cells to column 1 of a 96 well round bottom plate.
f. Transfer 200ul of parasitized cells to column 2 and dilute serially from column 2 to column 11.
g. Add 100ul of 2% RBCs on top of each well which was diluted serially; final volume in each well equals 200ul.
h. Add 200ul of 2% RBCs to column 12.
i. Spin plate in tabletop centrifuge at 1500 RPM for 5 minutes, low break.
j. Process this standard curve plate as other plates.
k. Make a new standard curve for each drug plate-strain combination. One standard curve plate can be used for all drug concentrations. The standard curve should look like the one below:



APPENDIX

APPENDIX A

Drugs*	Stock concentration	[Final]	Supplier
WR99210	20 μM in 100% DMSO	2.5 nM	Jacobus pharmaceuticals
G418/Geneticin	25 mg/ml in H_2O	0.5 mg/ml	Invitrogen
5-Fluorocytosine	5 mM	0.1μM	Sigma
Gancyclovir /Cytovene	10 mM	10 μM	Roche

*Store at -20°C

APPENDIX B

96 well smear
Manual technique
To smear a 96 well plate manually, resuspend the culture wells prior to smearing and, with a multichannel pipette, transfer 2-3ul on to a slide.

96-well automatic smear
Use custom 127.8 x 85.5 mm slides (Thermo Fisher Scientific).
Using the biomek: 1 μl of culture is dispensed at 0.1 mm above the slide surface. The tips then trace two concentric squares (0.33 and 0.66 mm wide) to smear the culture. Excess liquid is aspirated, and smears are allowed to air dry. A video of the process is available at: http://www.youtube.com/watch?v=2iX7utcDvnU

APPENDIX C

Blood Collection
Draw blood from the donor directly into the Whole Blood Leukocyte Reduction Filter Bags. Refrigerate blood for at least 4 hs at 4°C before Leukoreduction. Blood can be stored at 4°C for up to 3 weeks for malaria culture. After that period of time we consider the blood too "old" and get a fresh batch.

Leukoreduction

a. Work inside the Tissue Culture hood.
b. Soak scissors in EtOH 70% for 30 min.
c. Hang the bag at a higher level to allow flowing by gravity.
d. Cut the line to break open the barrier between bag and filter.
e. Collect the blood in 15-40ml aliquots in 50ml Falcon tubes.
f. Let serum and RBCs separate overnight at 4°C. If blood is needed immediately, spin to separate serum from RBCs for 20 min at 1500rpm, no break.

Washing blood

a. Put 5-10 ml of packed RBCs into 50 ml Falcon tubes and fill to 45 ml with RPMI wash.
b. Mix well pipetting or by gently shaking.
c. Spin down in tabletop centrifuge at 1500 RPM for 10 min at RT (Low brake).
d. Aspirate off the supernatant. Mix an equal volume of RPMI to the RBCs to make a 50% mixture.
e. Store in the fridge at 4°C for about a week.

APPENDIX D

Making media for malaria culture
Terry Minn and Manuel Llinás (04/17/02)
Updated by Edith Wong (10/10/02)

RPMIc. 0.5% Albumax in RPMI 1640 media (for 1 Liter):

a. Fill a beaker with less than 1 liter of double distilled water (ddH_2O).
b. Add 1 package of RPMI 1640 (GibcoBRL) into the stirring water.
c. Add 12.5ml of 2 M Hepes, pH 7.4.
d. Add 2g of sodium bicarbonate ($NaHCO_3$) powder.
e. Take 150 ml of this media and pour it into a separate beaker. Add 1 ml of 0.1 M hypoxanthine/1 M NaOH solution (1.361g hypoxanthine in 100 ml of **1 M NaOH**) to raise the pH of the media to 7.4. While stirring, add 5g of Albumax powder.
f. Combine the Albumax solution in with the previous media and mix well.
g. Finally, add 1 ml gentamicin (from UCSF Cell Culture Facility – 50 mg/ml).
h. Check the pH of your media. It should be around pH 7.4.
i. Bring volume up to 1 L with ddH_2O.
j. Filter the media into autoclaved 500 ml bottles using a 0.22 mm sterile Millipore filtertop. Use sterile technique and do this in the Biosafety hood!
k. Label the bottle well and include the date. Store at 4 ^{o}C.

Notes:

- *Media is good for only 2 weeks. When using media for culture, pre-warm in the water bath for 15 minutes.*

- *All solutions for making the media are stored at 4°C, except for the hypoxanthine/NaOH solution which is kept at room temperature and gentamycin which is stored at -20°C.*
- *This media is good for growing W2, 3D7, Dd2, and HB3 strains. For the Brazilian D6 strain, we have to add 5% Human sera and 5% high Albumax into the media.*

RPMI wash
Same recipe as RPMIc except for the hypoxanthine/Albumax addition step.

APPENDIX E

Synchronization method
Terry Minn and Manuel Llinás (04/11/02)
Updated for purposes of screen (7/18/08)

- This method is designed to eliminate the trophozoite and schizont stages hence selecting for ring-stage parasites.
- The most important thing for synchronization is to be sure that you have enough ring-stage parasites.
- For tighter synchronization, this method should be done several times until the ring-stage predominates in the cultures.

1. Make a 5% Sorbitol (sterile-filtered) solution. Pre-warm for 15 minutes in a 37°C water bath. (It can be stored long-term at 4°C.)
2. Remove the media from a T150 flask, with 50ml culture, using a Pasteur pipette on a vacuum line without sucking out any blood. (Until about 25.0ml of culture remains.)
3. Take out 8.0 ml of culture and do a smear on a microscope slide for analysis of parasitemia and to check the general synchrony of the culture.
4. Add 20ml of 5% Sorbitol to 50ml Flacon tube. Resuspend 25 ml of cells and transfer to the 50 ml conical tube, half filled with Sorbitol.
5. Mix well and incubate the cells 5 minutes at room temperature.
6. Spin the cells out of the Sorbitol solution in a tabletop centrifuge at 1500 RPM for 5 minutes, low brake.
7. Aspirate off the supernatant.
8. **Optional:** rinse cells of any remaining Sorbitol by resuspending pellet in 20mL of warm RPMIc and spinning in tabletop centrifuge at 1500 RPM for 5 minutes, low break. Aspirate off the supernatant.
9. Resuspend the cells in media such that when you add back to the flasks, the total volume of the culture is 50 ml.
10. Place back into the incubator.

APPENDIX F

SYBR Green Lysis Buffer
(Johnson 2006)

a. Add 2.423g Tris base in 1L bottle of cell culture water ($[20mM]_{final}$) and dissolve completely using a magnetic stirrer.
b. Adjust pH to 7.5 using concentrated HCl.
c. Add 10mL 0.5M EDTA ($[20mM]_{final}$).
d. Add 80mg saponin (0.008% w/v final).
e. Add 0.8mL Triton X-100 (0.08% w/v final).
f. Mix the solution thoroughly, avoiding the production of bubbles.
g. Vacuum filter the solution to remove particulate matter and store at RT. The solution is good indefinitely.

Note: We examined different concentrations of the various components of lysis buffer, but no significant improvement was observed.