KALADRUG-R: Laboratory SOP #10

Cloning of *L. donovani* isolates

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Annex documents: movies and ppt illustrating cloning procedures

Download the 5 following files in the SAME folder

http://www.itg.be/itg/dl/mp/P7160009.avi
http://www.itg.be/itg/dl/mp/P7160018.avi
http://www.itg.be/itg/dl/mp/P7160025.avi
http://www.itg.be/itg/dl/mp/P7160032.avi
http://www.itg.be/itg/dl/mp/cloning.ppt

A. Material required

1. 10 tubes with the medium used in your laboratory for parasite isolation; at ITMA, we use the Blood-agar Tobies (TSL) medium (see annex for recipe). Each tube is labelled before the cloning experiment with (i) code of the isolate to be cloned, (ii) number of passages [see SOP#13 for labelling of strains; should contain the number of passages since isolation of the 'mother' isolate and between brackets, the number 1 as this is the first passage from cloning], (iii) date of cloning and (iv) number of the clone (from 1 to 10)

   Example: On 14/02/09, the isolate BHU402/0 is cloned; at that moment it has been sub-inoculated 20 times since patient isolation; thus prepare 10 tubes labelled as following

   BHU402/0 R20(1) 140209 cl1

   BHU402/0 R20(1) 140209 cl2

   ...

   BHU402/0 R20(1) 140209 cl10

2. 10 tubes 0.5ml "conditioned Locke" (see below for preparation of "conditioned Locke")
3. 1 tube with 5ml of sealing medium, the monophasic medium (with serum) you use for growing *L.donovani* reference strain: this medium will be used for sealing the coverglass on the cavity slide
4. 10 tuberculin syringes with needle (26G x1/2)
5. Microscope with phase contrast objectives if possible (this allows a better visualisation of the parasite during cloning)

6. cavity slides (Brand; catn°: 475505), sterilized by autoclave
7. cover glasses, (24x24mm) wrapped separated in aluminum foil and sterilized by Pasteur oven (2hrs 180°C)
8. recipient with 70% alcohol to put in the used cavity slides
9. sterile glass micropipettes (+ device to cut the tip of the micropipettes)
10. Sterile cryogenic vials (Nalgene catn°:5000-1020) + liquid nitrogen resistant labels
11. 10 ML syringe + 19G ½ gauge needle

B. Operations before the cloning (preparing the isolate). D0 = planned day for cloning

1. D -14. isolate is taken out of the cryo 2 weeks before the cloning date; in preparation of the cloning experiment, we grow the thawed parasites on 2 tubes TSL
2. D -10. subculture of the 2 TSL on 2 fresh TSL
3. D -7 : subculture on 2 TSL or more if new cryo stabiltate is required (1.5 - 2ml Locke+ 0.5ml parasites)
4. D -3: subculture and if needed make cryostabilate (1.5ml Locke + 0.5ml parasite inoculum)
5. D -2: subculture on 1 TSL (1.5ml Locke + 0.5ml parasite inoculum)
6. D -1 : subculture on 1 TSL (1.5ml Locke + 0.5ml parasite inoculum) + prepare 10 eppendorf tubes with 0.5ml 'conditioned Locke' (see point C)
7. D 0 : cloning (10 clones) + subculture remaining in tube that was subinoculated at D -3 (this will serve as back-up if cloning fails)

C. Preparation of conditioned Locke

1. At D -7, fill 2 TSL with 8ml of Locke and put at 26°C for 1 week

LOCKE (OVERLAY TOBIE)	
bidistilled water	1000 ml
NaCl	8.0 g
KCl	0.20 g
KH_2PO_4	0.30 g
$MgSO_4.7H_2O$	0.10 g
$NaHCO_3$	1.00 g
Glucose	2.50 g
Penicillin	200,000 IU
Streptomycin	200,000 µg
pH adjusted to 7.4 with HCl or NaOH 1N	
Sterilize through a 0.22 µm membrane filter	

D. Cloning of *L. donovani* promastigotes (see also cloning.ppt, downloaded as instructed at start SOP)

The cloning is done in a sterile cabin, and all general rules of sterile working should be followed.

With the small flame of the Bunsen, make a very thin pipette (as thin as possible, but large enough to allow passage of fluid).

Make a pre-cloning dilution of the parasites in 2 or 3ml of "conditioned Locke": with a sterile pipette take a micro-drop of the parasites culture and put in a sterile tube containing 2–3ml of the "conditioned Locke", follow point 1-4 here below.

1. Put a drop of "conditioned Locke" solution in the cavity of the cavity slide.

2. Take 1 drop of the dilution with a micropipette and put it at the rear side of a sterile cover slide.

3. Put the cover slide over the cavity and seal it with a drop of sealing medium.

4. Take the cavity slide out of the sterile cabin and look at it under the microscope (object 16x) and check with the objective 25x and objective 40X.

If after 3 attempts (of previous steps 1 to 4) you never find any parasite in the microdrop, it means that your pre-cloning dilution is too high; in that case, add one micro-drop of the non diluted parasites to your pre-cloning dilution.

If after 3 attempts (of previous steps 1 to 4) you systematically find more than 3 parasites on your slide, it means that your pre-cloning dilution is too low; in that case, dilute it further with conditioned Locke. When you see 0, 1 or 2 parasites in the microdrop you can use the pre-cloning dilution for cloning.

5. When you see only 1 parasite in the drop, a second person has to confirm this (never lift the cover slide during this whole period).

6. Bring the cavity slide back in the sterile cabin.

7. Prepare a tuberculin syringe and take 0.2ml "conditioned Locke" out of the tube you have prepared.

8. Take the cover glass off of the cavity slide and put a drop out of the syringe on the micro drop.

9. Collect everything in the cavity, mix and put in a tube with TSL medium (or the biphasis medium you use for isolation).

10. Rinse the syringe with the remaining "conditioned Locke" solution of the 0.5ml tube.

11. Repeat starting from step 1 till 10 clones are found.

!!! See also the accompanying downloaded annex files (presentations and movies)

E. Maintenance and checking of the potentially growing clones

1. Incubate the tubes at 26°C and control every week for 4 weeks.

2. When positive, subculture onto TSL medium (1 ml Locke + 0.25 ml positive culture) if the culture is growing +++ make a stabilate (6 amp.) and a pellet (with the remains of the culture, centrifuge, wash a couple of times in PBS and centrifuge a last time, remove the supernatant and

keep the dry pellet frozen at -20°C; this may be used later on for DNA extraction, f.i. for checking the identity of the parasite frozen).

3. Don't forget to keep a very precise registration of the history of the clone (see SOP#13): it must be clear:

> on the reading sheet of each clone: (i) from which strain it was derived, (ii) the number of passages of the strain at the moment of cloning and (iii) the moment it was cryopreserved and the specific stabilate number.

> on the cryo-card of each clone: (i) from which strain it was derived, (ii) the number of passages of the strain at the moment of cloning and (iii) the moment it was cryopreserved and the specific stabilate number.

> on the flow chart of the strain: (i) how many clones were successfully derived from that strain (+ the number of passages at the moment cloning) and (ii) the stabilate numbers of each clone

Annex: TLS medium recipe

Tobie's Blood agar (Tobie E.J., 1949)	
bidistilled water	1000 ml
Bacto-Tryptose (Difco)	15.0 g
NaCl	4.0 g
$Na_3PO_4.12H_2O$	5.0 g
KCl	0.4 g
Bacto-Agar (Difco)	15.0 g g

pH 7.6, adjusted with HCl or NaOH 1N

NOTE: for soft agar : 2.0g Bacto-Agar / 1,000 ml

Recipe

2. Dissolve by boiling onto heating plate with magnetic stirring
3. Dispense per 80 ml in 250 ml screw-cap bottles
4. Autoclave at 121°C for 20 min and store in refrigerator
5. For use, melt in boiling water bath then cool down at 56°C for 30 min in a water bath
6. Add 20 ml rabbit blood obtained by aseptic heart puncture onto heparine (0.1 ml = 25 IU heparine/ml blood)
7. Mix gel and blood, dispense in tubes (±1 ml in 10x100 mm test tubes), slant
8. Incubate at 37°C for 24 hrs for sterility control
9. Add between 1 to 3 ml (depending on the strain) Locke or Locke-Krebs per tube just before use

NOTE: In case of cultivation in larger quantity, use bottles instead of test tubes:

Intermediate bottles (100 ml) : use 5 ml of gel-blood mixture

Large bottles (250 ml) : use 15 ml of gel-blood mixture

DO NOT slant the bottles