Bombardment

The protocol is based on previously published protocols (Praitis V., et al. (2001), Genetics., Berezikov E., et al (2004), Nucleic Acids Res., Radman I., et al (2013), PLOS One.)

Required materials and solutions:

- LB (enough for growing bacterial culture)
Calculations: for 1 construct you need to set up 1 worm pre-culture; for 1 pre-culture you need 300ml of HB101 (~OD600=1)
For feeding 1 worm-Culture (after worm pre-culture): ~2L of ON HB101 culture (Preferably have always at least 500ml more bacterial culture just to be on the safe side)
****grow HB101 plus 100ug/ml Str
**** Let Ana know in advance for excess use of LB

- Make sure you have **S-Basal** and all the other salts and cholesterol for worm culture (Volumes written below are for setting up 1 worm pre-culture/ 1 construct)

300 ml **S basal medium**
3 ml **1M potassium citrate, pH 6**
3 ml **trace metals solution**
0.9 ml **1M $CaCl_2$**
0.9 ml **1M $MgSO_4$**
0.3 ml **Strep (100 µg/ml)**
0.3 ml **Fungizone (2.5 ug/ml)**
0.3 ml **5mg/l Cholesterol**
20 ml **concentrated *E. coli* HB101**

8-10 ug of DNA (**plasmid, min 0.5 mg/ml)**
******* Preferably have concentrated DNA to keep the volume at min otherwise you can go up to 40ul to reach to the required amount)
or
20-40ug **(Fosmid , min 0.5 mg/ml)**

10 µl **2.5 M $CaCl_2$**
4 µl **0.1 M spermidine** (****keep always on ice, once you thaw, trash the remaining, DO NOT freeze/thaw repeatedly)
- 10 mg/ construct of 0.33–3 µm gold beads

- Check there are enough autoclaved-Baffled flasks and 500ml plastic buckets to pellet the bacteria.

- Check the Helium cylinder; make sure that the pressure on the manometer is ~ 2000psi when opened.

BRIEF:

1. **ALWAYS A DAY OR TWO BEFORE STARTING PRECULTURE START YOUR BACTERIAL CULTURE(2L/CONSTRUCT)**
2. **DAY BEFORE PRECULTURE, SETUP SMALL BACTERIAL CULTURE AND MAKE AN EGG PREP**
3. **PRECULTURE: MAKE 300ml BACTERIAL CULTURE (OD600=1) +20000 L1 WORMS**
4. **2-3 DAYS LATER, WORM CULTURE: 300,000-500000 MIX OF L1 AND EGGS IN 300ML SBASAL**
5. **2-3 DAYS LATER, BOMBARDMENT DAY**
6. **MAKE SURE TO HAVE ENOUGH DNA READY.**
7. **START PELLETING WORMS**
8. **WASH GOLD BEADS AND COAT WITH DNA AND VORTEX TILL BOMBARDMENT**
9. **BOMBARD THE WORMS AND LET THEM RECOVER FOR AT LEAST 30 MIN**
10. **DIVIDE BOMBARDED WORMS TO 30 PLATES**
11. **LEAVE FOR 48 HOURS WITH FOOD ON PLATE**
12. **ADD HYGROMYCIN TO THE PLATE**
13. **SCREEN AFTER A WEEK**

Step1:
Set up bacterial cultures:

**** You can always prepare bacterial culture well in advance and store the concentrated bacteria in -80C for up to 2 months (Bacterial culture does not need to be in dividing phase as they are used only as a food source)

Bacterial culture for worm pre-culture:
A day before setting up the pre-culture:
-Setup an ON culture
- dilute and grow to OD~0.9 (300ml/preculture)
*** You need to have the Bacteria in their growth phase for the worm preculture as you will not add more food to the preculture.

Bacterial culture for worm culture:
- Grow ON cultures
-Pellet at 4000Xg for 15min and keep pellets at 4C
- Re-suspend the pellet from 1L bacterial culture in 25ml LB and freeze at-80
**** Remember, you need to pellet at least 2L ON bacterial culture to be able to feed 1 worm culture through out the growth period

Step 2:
Preparing worms for pre-culture:

For each construct you need to start with egg prep from 1 large NGM plates full of healthy adults.
**** Make sure you have NO contamination, discard any plates that is contaminated; contamination will interfere with the growth of worms in Liquid culture.

-After egg prep, keep eggs ON only in M9 to have synchronized population of worms.

Worm pre-culture mix in **2L flasks**:
-20,000 L1s to each pre-culture (You can go as high as 40,000 worm/ pre-culture in such case later on make two worm culture from 1 pre-culture)
-5ug/ml cholesterol
- 300ml of ON HB101 culture(Explained above)
***** Work close to flame and keep the work area clean to prevent contaminations!!
***** Cover the flasks lid with two clean (preferably autoclaved, aluminum)

Keep the worm pre-culture for 2-3 days at 20C environmental room, while shaking (at number 4 setting for Hobert shaker and at 185rpm if Greenwalds shaker is being used).

***Take small aliquots every day from the preculture to screen the growth of worms and to check for any contaminations.
The idea is that the synchronized L1 worms grow to gravid adult during the incubation time and lay eggs in the preculture. The bacteria that was added on the first day to the preculture, since was on growth phase will grow slowly and is just enough amount for worms to grow and lay eggs.
Once you have loads of L1 worms in the preculture, it is ready to set up a worm culture.
**** Do not try to have synchronized population, mix of eggs, L1 and L2s (preferably 60% L1) are perfect for starting the culture.

Step 3:
Preparing worms for culture:

Once the preculture is ready, tilt the flask and let it stand for 15 min next to the flame. You need the supernatant, as larvae are light and will not settle in 15 min; majority of adults will settle and you can pipette out the worm pellet (Either trash them or if you have more bombardment coming up, plate the worms and use the next generation to start up another pre-culture).
***** Remember that growing and swimming in the liquid culture is stressful for worms. Do not keep worms in the liquid culture for more than 2 generations.

- Use centrifuge bottles to pellet the worms from the culture
- Spin 990X g/ 3 min
- Take an aliquots and count the number of larvae in the pellet

If you have started preculture with 20,000 worms, now you must have at least 500,000 worms to set up your worm culture. You can go as low as 300,000 worms for starting the worm culture.
***** These numbers are all for bombardment of ONLY 1 construct.

- **Prepare the media for worm culture (each culture must be in <u>2L flasks</u>):**

- 300 ml S basal medium
- 3 ml 1M potassium citrate, pH 6
- 3 ml trace metals solution
- 0.9 ml 1M $CaCl_2$
- 0.9 ml 1M $MgSO_4$
- Strep (100 µg/ml)
- Fungizone (2.5 ug/ml)
- 5mg/l Cholesterol
- 25 ml concentrated *E. coli* HB101

Add 300.000 to 500,000 L1s and keep the culture for 2-3 days at 20C environmental room, while shaking till worms are **late L4 to early adult** (40%-60% ratio)

(shake at setting number 4 for Hobert shaker and at 185rpm if Greenwalds shaker is being used).
***It is extremely important to take small aliquots every day from the culture to screen the growth of worms. Once worms are at L3 to L4 stage, need loads of food. Mostly from second day onward you need to add another 25ml of concentrated bacteria.
***** Do not let the worms to starve before bombardment and do not add too much bacteria to the culture as if the culture is too concentrated will cause worms to suffocate!

Day before bombardment:

- For each bombardment take one uncoated-medium size plates and leave at 37C for few hours to have the plates dry to have higher absorbance capacity
- You can wash the gold beads a day in advance and keep at 4C but DO NOT coat them with DNA or they can be washed on the day of bombardment
- Make sure Helium cylinder is filled
- Check that the stopping screens are clean and autoclaved (1 screen/ construct)

Step 4: DAY OF BOMBARDMENT
Preparing worm:

-Tilt the flask, close to the flame, and let the worms to settle for 20-30 mins
- Using 25ml pipette, take out the worms settled at the bottom of the flask to 50ml falcon tubes. Make sure you are taking all the worms to get max number of worms.
-Centrifuge at 800 rcf, 30 sec (You should have from 3-5 ml of worm pellet from pelleting 1 worm culture)
- Discard the supernatant and add 15 ml fresh S basal and let the worms on the nutator at 20C till bombardment.
**** If worms are running out of food make sure to add a bit of bacteria not to have the worms starved.
*****Transfer the 9cm plates that you dried yesterday to cold room to have them cold for the time of bombardment to prevent worms from crawling and clustering.

Preparing gold particles:
Gold bead preparation

- A total of 10mg of 0.33–3 μm gold beads were used for each construct (you can weight up to 60mg in one 1.5 ml eppendorf tube and continue as follow)

* the volumes mentioned below are for one eppendorf tube no matter there is 10 or 60mg gold in it.

- Add 1 ml of 70% ethanol

- vortexed for at least 5 min.
- Allow beads to settle for 15 min
- Centrifuge for 3–5 sec at 100 rcf
- The supernatant was aspirated off and the following steps were repeated three times:

– 1 ml of ddH_2O was added and the tube was vortexed for 1 min;
– Tubes were incubated at room temperature for 1 min to allow particles to settle;
– Samples were spun down briefly (3–5 sec) in microcentrifuge and the supernatant was removed;
– 160 µl of 50% glycerol was added per each 10mg gold to bead pellet.
***** Washed gold beads can be kept at 4C for up to 2 weeks.

DNA loading onto gold beads

- Vortex beads in 50% glycerol for at least 5 min
- Aliquot 150µl gold beads/ construct into low adhesion tubes (Eppendorf) and the following solutions were added to each bead aliquot in the order listed:
***** VERY VERY IMPORTANT TO KEEP BEADS VORTEXING WHILE ADDING THE FOLLOWINGS.

- 60 µl 0.1 M spermidine, vortex 1min
- 8-10 ug DNA(~ 1ug/ul) or 20ug fosmid (**** if you want to co-bombard different constructs, the total amount of mix DNA should fall again within the same range example: 1 ug of Hygromycin plasmid+ 7-9ug of desired DNA (total 8-10 ug) OR 1ug hygromycin plasmid+19 ug of fosmid (total 20ug)
- vortex, 1min
- 150 µl 2.5 M $CaCl_2$
- Vortex for 5 min
- Allow beads to settle down for 1min
- spin for 30 seconds on bench-top centrifuge
- Carefully discard supernatant with out disturbing the pellet (DO NOT use vacuum)
**** If there is a little amount of gold in the meniscus, just tilt the tube and slowly pipette out the supernatant.
-Resuspend in 450 ul 70% EtOH
**** Gold beads might be stuck to the wall of the tube, carefully and gently scrape off with the pipette tip while adding EtOH.
- Pipette up and down till you get everything resuspended. When using fosmid it takes longer to resuspend the beads back to solution. Be patient!!! You should not have gold chunks!!! When you tilt the tube should see a very fine streak of gold.
- Spin for 30 sec on bench-top centrifuge
- Carefully remove supernatant and resuspend the pellet in 150 ul 100% EtOH
DNA-bound beads must be then continuously vortexed until loaded onto macrocarriers otherwise the DNA will precipitate!

Bombardment:

time	bomb1	bomb2	bomb3	bomb4	bomb5	bomb6	bomb7	bomb8	bomb9
00:00	isopropanol								
00:05	dry								
00:10	worms								
00:15		00:15 isopropanol							
00:20	DNA	00:20 dry							
00:25		00:25 worms							
00:30		00:30	00:30 isopropanol						
00:35		00:35 DNA	00:35 dry						
00:40	bomb	00:40	00:40 worms						
00:45		00:45	00:45	00:45 isopropanol					
00:50		00:50	00:50 DNA	00:50 dry					
00:55		00:55 bomb	00:55	00:55 worms					
01:00			01:00	01:00	01:00 isopropanol				
01:05			01:05	01:05 DNA	01:05 dry				
01:10			01:10 bomb	01:10	01:10 worms				
01:15				01:15	01:15	01:15 isopropanol			
01:20				01:20	01:20 DNA	01:20 dry			
01:25				01:25 bomb	01:25	01:25 worms			
01:30					01:30	01:30	01:30 isopropanol		
01:35					01:35	01:35 DNA	01:35 dry		
01:40					01:40 bomb	01:40	01:40 worms		
01:45						01:45	01:45	01:45 isopropanol	
01:50						01:50	01:50 DNA	01:50 dry	
01:55						01:55 bomb	01:55	01:55 worms	
02:00							02:00	02:00	02:00 isopropanol
02:05							02:05	02:05 DNA	02:05 dry
02:10							02:10 bomb	02:10	02:10 worms
02:15								02:15	02:15
02:20								02:20	02:20 DNA
02:25								02:25 bomb	02:25
02:30									02:30
02:35									02:35
02:40									02:40 bomb

* Based on the table above you can simultaneously handle few bombardments all in much shorter time!

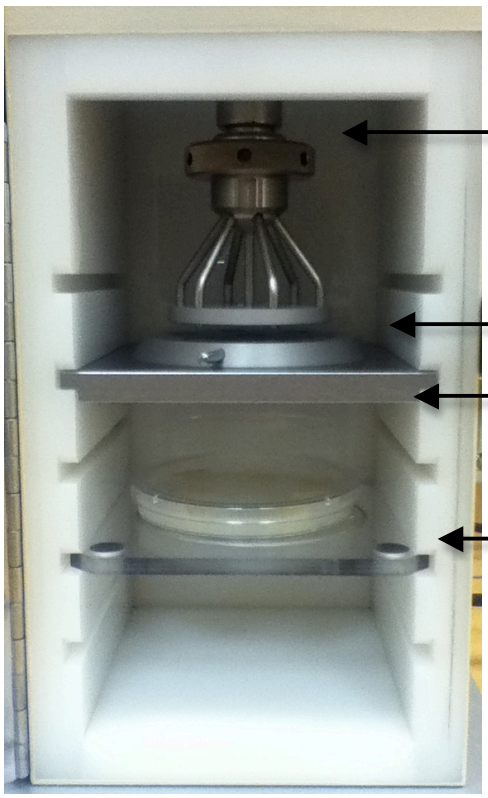
- Clean the working area not to have dust on the macrocarriers
- Soak seven macrocarriers/ construct in HPLC-grade isopropanol, 5 min
- Let them dry out for 10 min on a piece of whatman paper
- Meanwhile pellet the worms that you have prepared earlier by centrifuging them at 800rcf for 30 sec.
- Discard the supernatant
- Close to the flame, gently and evenly spread 3 ml of worm pellet to the dried-cold 9cm plate; gently move the plate to have an even layer of worms covering the whole surface

  *** plate ~200ul of worm pellet on 1-2 small plates and keep it aside for later on as an control (DO NOT bombard these plate!!)
- Let the plate with the lid open next to the flame till the plate absorbs all the liquid.

**** you do not want to have worms swimming or super dried, normally takes 15-20 min.

- Turn the macrocarriers concave side up and load 20ul of DNA-coated gold beads to the centre of macrocarriers (*** keep pipetting up and down to make sure you keep a homogenous solution)

- Let the DNA to dry on the macrocarriers while the worm plate is also getting ready.



Bombardment was carried out as described in the product manual for the Biolistic PDS-1000/He (BioRad Laboratories Ltd.).
http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_9075.pdf
***** Keep the distance between each compartment as shown in the picture above.

- Insert the rupture disc into the retaining cap and make sure it is perfectly fitted using torque wrench so that even if you turn the cap upside down the disc won't fall.
- Screw the retaining cap tight otherwise the vacuum will not retain in the chamber
- Insert macrocarriers into the hepta-adapter (gold-coated side up), gently push the edges down with red seating tool.
- Place the stopping screen on top of the macrocarriers in the hepta adapter and put the adapter in the chamber
- Place the plate (DO NOT forget to remove the lid)
- Close the door of the chamber
- Turn on the vacuum pump and open the helium cylinder
- Wait till the vacuum reach to 27 inHg
- Quickly flick the switch to hold position while pressing and **holding** the fire switch

**** These two switch need to be flicked **simultaneously and very quickly**, make sure the vacuum hold at 27inHg, otherwise start all over again!!

- Hold till the rupture disc blows at 1100-1400 psi
- Press vent switch, allow pressure to reduce, take the plate out and let worms to recover for 30 min
- Add 5 ml of M9 to the plate and wash the worms off the plate
- Equally divide the volume into 30 seeded small plates (As you increase the number of plates you will have higher chance to get more independent lines. From now on treat each plate as one line)
- Let plates at 20C for 48h

Post-bombardment worm maintenance and selection:

-Always include a plate with non-bombarded worms (You prepared this plate when you were plating worms to bombard) to see that the selection works. Based on the control plate you can tell when all non-transformed worms are dead and start screening (though it can help to wait a bit longer to get nice populations).

-Leave the animals to lay eggs for max. 48h(ideally the plates will be full of arrested L1s at this point)
-add hygromycin B to a final concentration of 0.3 mg / ml
- Dilute the hygromycin in/with Strep/fungizone to make pipetting/spreading easier and to reduce contamination.
- If plates are out of food, don't care. Add 200ul of concentrated bacteria on the second day after adding hygromycin
- Start screening after 1 week
*** Hygromyccin is most obvious after the fifth day
**** Be careful Hygromycin half-life is long but the effect is reversible
- Majority of positive plates should be true lines but just to be on the safe side, transfer a chunk from all the plates to fresh hygromycin plates to rescreen after another 1-2 weeks.
*** Strains with high transmission rates should establish populations quickly and be the first to come up. The rescreening is to find lines with low transmission rates where populations come up later. Non-transformed dauers are very resistant to hygromycin and take very long to die, they can however not exit the dauer stage in the presence of antibiotic and so they aren't really a problem.