Zebrafish BAC Recombineering (Burns Lab)

Preface

This guide provides detailed instructions for identifying and modifying bacterial artificial chromosomes for zebrafish transgenesis. Most of what is included was derived from the NCI Frederick National Laboratory Website (NCI) and Warming *et al.* (Warming et al., 2005). The ATI, ITK, and listed targeting constructs were generated in our laboratory (Zhou et al., 2011). Another great source of information on this subject comes from the Kawakami laboratory (Suster et al., 2011).

Please don't hesitate to contact me directly with questions at gburns@cvrc.mgh.harvard.edu.

Best,
Geoff Burns (January 2013).

Finding a BAC

There are generally two places online to identify BACs containing cis-regulatory elements of interest.

1. ZFIN (http://www.zfin.org) – Click on "Genes/Markers/Clones" and enter gene name or symbol in the in "Name/Symbol contains" field. Choose "gene", not "BAC" from "Types" menu. You will get one of three outputs from your search:
    a. A list of hyperlinks to gene pages matching your search term (for instance, if you insert "fgf"). Click on the appropriate link to bring up the gene page of interest.
    b. A hyperlink to the actual gene page of interest (for instance, if you insert fgf8a).
    c. a message stating "gene search results (0)" (for instance if you enter "nothing"). In this case, move to option 2 below.

    Click the hyperlink for the gene page of interest and look for the subheading "Segment (Clone and Probe) Relationships". Here, you might find an entry for a BAC containing your gene of interest such as "gene fgf8a contained in: [BAC] CH211-194I8". Click on the BAC name link to see more information about the BAC. Most importantly, use the schematic to determine how much genomic sequence is upstream and downstream of the first coding exon. In our experience, 20Kb+ of upstream and downstream sequence will have a good chance of recapitulating endogenous expression patterns. The source of the BAC and a link for ordering is also provided on this page.

2. Sanger center *D. rerio* blast server (I have included an example of how we found an *nkx2.5* containing-BAC on page 23)
    a. First, retrieve a full-length cDNA sequence for your gene of interest. A link to a potential full-length cDNA sequence can be found on the http://www.zfin.org/ gene page under the subheading "sequence information". It will be located in the accession number column in the same row as RNA. Alternatively, searching the NCBI Nucleotide Database (http://www.ncbi.nlm.nih.gov/nuccore) can also quickly yield a full-length cDNA sequence. For better searching, type the word Danio before your gene name or symbol in the search field.
    b. Go to the Sanger Center *D. Rerio* blast server (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/d_rerio). Paste your cDNA sequence into query data field and start blast.
    c. A blast server results page (without any results) will appear. Leave the format pull down menu on "graphical". After a couple of minutes click the retrieve tab and if the search is complete the results will appear below on the same page.
    d. Scroll past the color diagram and list of blast results to the first couple of hits. For each hit, there will be a graphical representation of the BAC showing its size and where in the BAC and one or more alignments

between your cDNA query sequence and the BAC hit. Although several different BAC hits will be shown, typically not more than a few listed hits are real hits. Real hits will have near perfect alignments and identity scores in the mid to high ninety percent range. Scattered mismatches in these alignments indicate polymorphisms. False hits will have many more mismatches and much lower identity scores.

If you have more than one true hit, check the sizes of the genomic sequences surrounding the locus of interest. This can be done by looking both at the graphic and the orientation of the query relative to the hit in the alignments. A query with increasing numbers from left to right and a BAC with increasing numbers from left to right indicate that the 5' end of the locus is to the left. However, a query with decreasing numbers left to right and a BAC with increasing numbers left to right indicates that the 5' end of the locus is to the right. Once the orientation of the locus in the graphic is understood, the relative sizes of the upstream and downstream regions should be relatively easy to ascertain.

We generally choose a BAC with 20Kb+ of genomic sequence surrounding the coding sequences for the gene of interest. Once you decide on a BAC, click the EMBL identifier located above the BAC graphic. This will pull up a new page. Under the description subheading, you will see the BAC identifier.

The most common zebrafish BAC hits will come from three libraries, the DanioKey, CHORI-211 library, and CHORI-73 library, although you might find hits in other libraries. The specifics of each library are found here: http://www.sanger.ac.uk/Projects/D_rerio/library_details.shtml

BACs in the CHORI-211 (http://bacpac.chori.org/zebrafish211.htm) and CHORI-73 (http://bacpac.chori.org/library.php?id=281) library can be ordered (http://bacpac.chori.org/ordering_information.htm) from CHORI (http://bacpac.chori.org/library.php?id=281).

BACs from the Danio Key library were originally available through Imagenes/RZPD, now Source Biosciences Life Sciences. At the time of this writing, it doesn't appear from their website that Danio Key BACs are available through this company.

When the BAC arrives, streak it out to single colonies on LB + chloramphenicol (for final concentrations of antibiotics, see pg. 22). Grow cultures overnight at 37ºC and perform BAC minpreps and diagnostic digests.

BAC miniprep protocol

1. Grow 5 ml LB cultures (+/- antibiotics depending on what you are growing; final concentrations listed on pg. 22) in 14 ml round-bottom Falcon tubes overnight in a shaking incubator. For bacteria containing BACs from the distributor, growing at 37ºC is optimal. **If growing SW105 cells (with or without BAC), the temperature should not exceed 32ºC.**

2. Pellet the bacteria and pour off supernatant.

3. Resuspend pellets in 250 µl Qiagen Buffer P1 (50 mM Tris·Cl, pH 8.0, 10 mM EDTA, 100 µg/ml RNase A). The total volume at this stage will be a little greater than 250µl because of residual supernatant. Transfer 250µl of the resuspension to Eppendorf tubes and save what remains in the culture tubes at 4ºC.

4. Add 250 µl Qiagen Buffer P2 [200 mM NaOH, 1% SDS (w/v)], mix by gently inverting tubes several times, incubate at room temperature for ~5 minutes.

5. Add 250 µl Qiagen Buffer N3 (composition proprietary) or P3 (3.0 M potassium acetate, pH 5.5), mix by gently inverting tubes several times.

6. Microfuge tubes on high for 10 minutes at room temperature.

7. Transfer supernatants to new Eppendorf tubes.

8. Add 750µl isopropanol, mix by inverting several times, and chill tubes at -20ºC for at least 20 minutes (or indefinitely until you get back to finishing the prep).

8. Spin tubes in microfuge on high for 10 minutes at room temperature. Pour off supernatant.

9. Gently add 1ml 70% EtOH, invert gently a couple of times, spin for 1 minute, pour off wash, pulse spin, remove residual wash with a pipet, and let the pellet airdry with tube upside down at room temperature.

10. Resuspending the pellet:

If the BAC will be transformed into SW105 cells, resuspend pellet in 40µl H_20 and proceed to **Electroporation of SW105 cells**.

For diagnostic digests, resuspend each BAC pellet in 40µl pre-mixed digestion solution. For multiple pellets, make a master mix.

Per reaction: 34µl H_20
4µl 10X buffer
2µl Enzyme
40 µl Total

Usually, a pellet is visible at the bottom of the tube but also splayed up one side. The DNA pellet can be resuspended completely by pipetting liquid on the side of the tube. Before incubating the digestion, pulse tubes in microfuge to collect sample at the bottom.

11. Incubate digest for 30-60 minutes at 37ºC.

12. Run entire 40µl digest (no need to save any) on 0.6% TBE gel and compare actual banding pattern with predicted banding pattern. The predicted banding pattern comes from a virtual digest of the BAC sequence.

Notes:

1. For confirming the BAC identity for the first time, *SpeI* is a good enzyme because the banding pattern is usually not too complex to decipher.
2. Often, you won't be able to see all of the lower MW bands (<1Kb) and it won't be possible to precisely size the largest bands (>12Kb). However, it should be possible to confirm the BAC identity by looking at the bands between 1Kb and 12Kb. You might find some slight deviations between the predicted pattern and actual pattern. These can often be explained by the lack of the BAC backbone in the sequence file.
3. It might be possible to determine the orientation of the BAC sequence with respect to the library backbone based on the banding pattern. One of the easiest ways to do this is to create two maps, one with the insert in the forward direction and one with the insert in the reverse direction. Whichever virtual digest matches the observed banding pattern will give you orientation.
4. Sometimes, even when taking the backbone into account, you might find slight deviations (one band for instance) from the predicted banding pattern. This could result from inaccuracies in the BAC sequence. Ultimately, you will have to use your judgment to determine if you have the correct BAC.

Obtaining SW105 cells

We will be happy to provide the SW105 cells if you contact me with a shipping address and FedEx number.

However, before we can send the strain, you will need to execute a Simple Letter Agreement through the NCI Frederick website.

To do this:

Create an account here: http://ncifrederick.cancer.gov/Research/Brb/logon.aspx.

Login, accept the terms, click recombineering, choose 1 bacteria set, add to cart, proceed to checkout, next, previous SLA on file (no), follow instructions to fill out SLA.

After its executed, let me know, and we can send out the bacteria.

At the time of this writing, the NCI appears to not have any more aliquots of the bacteria.

Electroporation of SW105 cells with the BAC or targeting construct

You will follow the same protocol for transforming the BAC into SW105 cells or transforming the targeting construct into SW105 cells already containing the BAC. When transforming a targeting construct, there is a heat shocking step and an additional sample.

Preparation of cells

Goal: to replace the LB with chilled water because any residual salt will compromise the electroporation. Everything should remain cold (on ice or in refrigerated centrifuge) at all times except for short periods when pouring off/removing supernatant and adding cold water to the tubes. During these steps, the amount of time the tubes are off ice should be minimized.

1.) Grow 5ml LB cultures (- antibiotics for SW105 cells not containing BAC, + Chl for SW105 cells containing BAC, + Chl and Amp for SW105 cells containing BAC with ATI, + Chl and Kan for SW105 cells containing BAC with ITK) in 14 ml round bottom polypropylene Falcon tubes shaking overnight at **32ºC**. Inoculate culture from a glycerol stock, single colony, or previous culture stored at 4ºC.

2.) The following day, make 30 ml LB with the same antibiotics used for the overnight culture. Transfer 25 ml into a 250 ml Erlenmeyer flask. Save the extra for blanking the spectrophotometer. Let culture shake at 32ºC for a few minutes. Take OD_{600}. It should be approximately 0.1-0.2. If greater than this, dilute to this range. If less, and time is an issue, add more starting culture to increase OD to 0.1-0.2 range. If time is not an issue, just let the culture grow at 32ºC. Check OD periodically.

3.) Prepare for electroporation after starting the culture:

a) Begin cooling centrifuge. We use an Eppendorf 5810R table top centrifuge with an A-4-44 Rotor and buckets capable of holding two 50 ml Falcon tubes (cat. #5804 706.005). I set the temperature to -4ºC. It never gets this cold, but setting it below 0 has the best chance of keeping it as cold as possible. About 15 minutes (during the heat shock if appropriate) before spinning the cultures, fastcool the centrifuge. On our machine, this lowers the temperature a few degrees more.

b) Begin chilling water and tubes. For each electroporation, you will need ~25 ml pre-chilled autoclaved H_20, a pre-chilled 14 ml round bottom polypropylene Falcon tube, a pre-chilled eppendorf tube, and a 0.1cm electroporation cuvette (BioRad Cat#165.2089) . For each recombineering reaction, you will have one “induced” sample and one “uninduced” sample so multiply everything above by two and label the tubes and cuvettes accordingly (U=uninduced; I=Induced).

Only necessary for BAC recombineering, not BAC transformation:

c) Turns on heater in shaking water bath. Set to 42ºC.

5.) When the 25 ml culture reaches an OD of 0.55-0.6 (generally 1.5-2.0 hours after starting the culture), it is time to start the transformation procedure.

BAC transformation (no heat shock): pour 10 mls of culture into pre-chilled 14 ml round bottom polyprolylene falcon tube and keep tube on ice. Proceed to Step 6.

BAC Recombineering (heat shock): pour 10 mls of culture into pre-chilled 14ml round bottom polyprolylene falcon tube and keep tube on ice. Place the flask with the remaining culture in the pre-warmed shaking water bath. Immediately start a timer for 15 minutes after the flask bottom is submerged. Initiate shaking. Very slow shaking is adequate.

After exactly 15 minutes, immediately remove the flask from the shaking water bath and place on ice. Pour 10 mls of heat-shocked culture into pre-chilled 14 ml falcon tube and keep tube on ice. Proceed to Step 6.

6.) Pellet cells in the pre-chilled table top centrifuge. In our setup, we can get the majority of cells to pellet after 3' at 5000 rpm. The goal is to spin the cells down as fast as possible in a chilled environment. For another setup, the optimal conditions will need to be determined empirically.

7.) Make an ice water slurry in an ice bucket.

8.) After the spin is complete, remove tubes from centrifuge and put back on ice. Start the centrifuge fastcooling again. Spread out a paper towel. For each tube separately, pour off the supernatant and, with the tube upside down, dab the rim of the tube top on the paper towel several times to remove the residual supernatant. As the dabbing continues, the supernatant absorbed by the paper towel each time will diminish to almost nothing. Put the tube back on ice. This should take 5-10 seconds.

9.) Add 1 ml ice-cold autoclaved water to each 14ml tube. Add it to the side of the tube so as not to disturb the pellet.

10.) Putting up to two tubes in one hand, submerge the bottom of the tubes in an ice water slurry and swirl at ~120 rpm until the pellets are completely resuspended. Check the pellets by removing the tubes from slurry and quickly checking for the continued presence of a pellet. The pellet should get continually smaller. This can take several minutes the first time. It is possible that the pellets will come off the bottom of the tubes. Just keep swirling until the pellets are resuspended. Once both pellets are completely resuspended add 9 mls ice-cold autoclaved water to each tube (again, add the water to the side of the tube) and place back on ice.

11.) Repeat steps 6-9, the pellet should resuspend faster the second time.

12.) Spin a third time. This time, don't pour off the supernatant, but instead remove 9 ml with a serological pipet leaving ~1 ml (just eyeball it) in the tube. Resuspend the pellet by swirling again in the ice-water slurry.

13.) Transfer the 1 ml of resuspended cells to the pre-chilled eppendorf tubes.

14.) Pulse spin the eppendorf tubes to pellet all bacteria. This can be done by quickly pulsing at RT. Better to use a refrigerated centrifuge. Put tubes back on ice.

15.) Remove all but approximately 100µl of the supernatant with a p1000. Again, just eyeball it.

16.) Add DNA to each tube:

If transforming SW105 cells with a new BAC: use 5-10µl of a BAC miniprep. Spec'ing the BAC DNA will not give an accurate reading because the BAC miniprep is so dirty. Better yet, do two transformations, one with 5µl and another with 10µl.

If transforming a targeting construct, add up to 4µl maximum volume of DNA eluted in water (see section on preparing the targeting construct). The goal is to maximize the amount of DNA up to 1µg. Therefore, add 1µg (in less than 4µl) or add 4µl (less than 1µg).

16.) After adding the DNA, resuspend the pellet using a p200 and transfer everything to the gap between the metal plates in the prechilled electroporation cuvettes.

17.) Electroporation. We use the BioRad Gene Pulser system and the following settings: 1.7kV, 25µF, 200ohms. After setting the machine, press the time constant button. Insert the cooled cuvette into the electroporation slider. Press and hold the "pulse" buttons simultaneously until the electroporator beeps, remove the cuvette from the slider, and immediately add 1 ml room temperature SOC media. On our apparatus, a respectable time constant is 4.7. A time constant that is a few tenths less than 4.7 is OK, but a very low time constant (below 4.0) indicates that the cells will likely not survive the electroporation.

18.) Pour the electroporated cells from the cuvette into a fresh 14 ml round bottom polypropylene tube and let them recover in the 32ºC shaking incubator for 1 hr.

19.) After recovery, transfer the cells to an eppendorf tube, pellet in a microfuge, remove all but approximately 100µl SOC. Resuspend pellet in residual SOC and plate all on appropriate antibiotics.

20.) Incubate plates at 32ºC overnight. A successful recombineering will produce many more colonies on the induced plate compared to the uninduced plate. If there is not an enrichment on the induced plate, then the background is too high and the targeting construct will need to be reprepped. Try lowering the template present in the PCR and make sure to *DpnI* treat the PCR reaction thoroughly.

21.) Pick several colonies from the induced plate and inoculate 5 ml cultures of LB containing the appropriate antibiotic(s). Also, grow a 5 ml culture of the parent strain. Don't forget that your parent BAC will not be resistant to the selectable marker in your targeting cassette. Miniprep according to the "BAC miniprep" protocol and perform diagnostic digests as described. Generate a sequence file with the modified BAC and choose an enzyme that will allow for determining if the recombineering worked as intended.

PCR amplification of the targeting construct

Designing primers
For every recombineering reaction, you will need a pair of primers and the PCR template. Various PCR templates can be obtained from our laboratory (see list on pg. 20).

We generally replace the coding sequence in the first coding exon with a cDNA of choice (GFP, cre…).

Design two primers according to the following guidelines. No modifications to the primers are required during synthesis.

Forward primer = 40bp homology directly upstream of the endogenous ATG + 5' primer binding site in plasmid template*.

Reverse primer = reverse complement of 40bp homology immediately downstream of first coding exon (including the splice donor site GT) + reverse complement of 3' primer binding site in plasmid template*.

*I can provide this information when providing the template with map. For the forwad primer, the primer binding site in the plasmid templates will generally include a Kozak consensus sequence. If not, then best to include one (ACC) in between the homology region and template-binding region.

PCR amplification of the targeting construct
Enzyme: It is best to use a high-fidelity enzyme like Phusion (NEB). Set up a trial reaction (usually 50µl). If it works well, then scale it up 11X. If it doesn't work well, we use the Failsafe PCR system (Epicentre). I try the first 11 buffers. If most work, then you can proceed directly to purifying the PCR product. If only a small number work, set up the reaction again 11X with the buffer that worked best.

Template: I use 1µl of our maxi stock (whatever the concentration might be) across all 11 reactions.

Do the PCR and load each reaction (50µl/per well) across a 12-well gel (one well left for ladder).

QIAquick Purification of the targeting construct
If the PCR worked well, then you should see a prominent band of the correct size in each of the wells. Cut out all 11 bands in one horizontal gel slice.

Perform Qiaquick gel purification according to the manufacturer's protocol [(http://www.qiagen.com/products/dnacleanup/gelpcrsicleanupsystems/qiaquickpcrpurificationkit.aspx)]. Divide the dissolved agarose across four columns. Sometimes the gel slice will be bigger than what they recommend per column (400mg agarose). If your gel slice exceeds their recommendation, then you can add 750µl QG to the columns between

the DNA loading and wash step. This will remove any residual agarose as a result of overloading the column.

After washing the columns with QC, elute each column in 40µl H_20 and combine all four into one tube. The total volume will now be approximately 150µl, but you should measure it with a p200.

DpnI treatment
This step removes any remaining template in reaction that will show up as background on the plates.

To your eluted DNA, add 20µl NEB buffer 1,2,3, or 4 and 5µl *DpnI* enzyme and bring up the total volume to 200µl with H_20. Incubate at 37ºC for ~30 minutes.

Minelute purification and concentrating the targeting construct
Perform MinElute purification (Qiagen) of the digested DNA (no need to rerun on gel).
To your digest, add 600µl QG buffer and 200µl isopropanol
Add 750µl to one minelute column. Spin.
Add remaining to the same column. Spin.
Add 750µl wash buffer QC. Spin.
Remove wash, spin again in same tube.

VERY IMPORTANT – at this stage you might think that all of the wash has been spun from the column. I have found that this is not the case. And any residual wash will interfere with the spectrophotometer reading of the DNA and you might think you don't have any DNA when, in fact, you have plenty. Therefore, after the second wash spin, transfer the column to a fresh Eppendorf tube and spin again. Look at the bottom of the tube for additional wash solution that has eluted from the column (it might even be a size of a pinpoint). Repeat this several times until you don't see any wash solution spinning out of the column. Even after you don't see any wash coming off, I would advise spinning a couple of more times just to be safe.

Elute in 12µl H20.
Spec 2µl on nanodrop.
Transform up to 1µg targeting construct per transformation (see above).

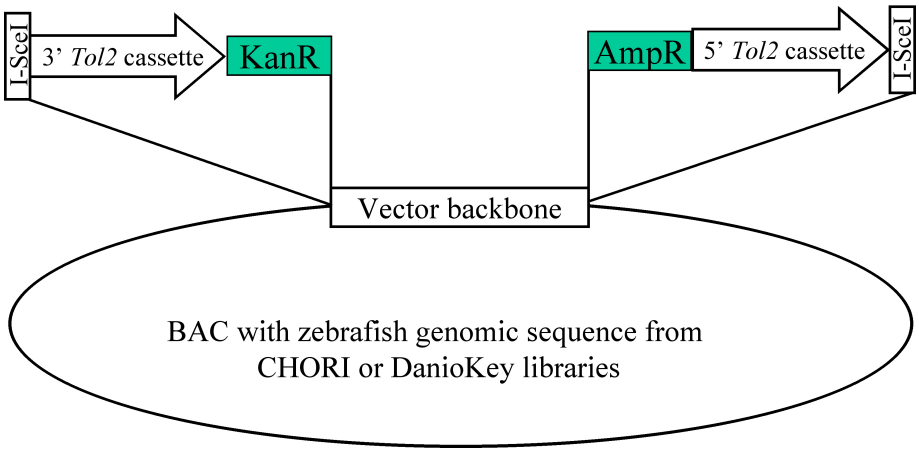
Excising KanR cassette from BAC containing targeting construct

All of our targeting cassettes contain a Frt Kan Frt cassette downstream of the cDNA that is being inserted. This cassette is surrounded with Frt sites and can be excised using arabinose inducible Flp recombinase in SW105 cells. It is preferable to remove the Kan casssette to minimize any adverse effect on transgene expression. Removing the KanR cassette is also necessary if you are adding the ITK cassette.

1. Grow 1ml culture of SW105 cells with correctly recombineered BAC to OD600=0.55-0.6. Add 10 μl of 10% arabinose stock (1g in 10 ml H_20; make fresh) and let culture grow for 2-3 hours.
2. Dip a p1000 tip into the culture and streak to single colonies. Incubate plate overnight at 32ºC.
3. The next day, pick 4 colonies and test them for KanR. To do this, inoculate 4, 1 ml cultures (LB + Chl **but minus Kan!**) with four individual colonies. Let grow for a few hours or overnight.
4. Spot 10 μl of each culture on one LB –Kan plate and one LB + Kan plate (you can easily fit four spots on one plate). Grow spotted plates overnight.
5. A Kan sensitive colony will not grow on the Kan+ plate but should on the Kan- plate.
6. At this stage, it is advisable to maxi prep a correctly modified BAC and inject to determine if the transgene is expressed in the cells/tissue of interest before performing ATI and ITK modifications.

Surrounding BAC insert with Tol2 and ISceI containing cassettes ATI and ITK

To increase the efficiency of germ-line transmission, you might consider surrounding your BAC insert with *Tol2* and *ISceI* sites. For this purpose, we have designed two cassettes, AmpR, Tol2, ISceI (ATI) and ISceI, Tol2, KanR (ITK) (see schematic below).



We have successfully added ATI and ITK to Danio Key BACs using the following template/primer combinations. The underlined portions are the 40bp of homology to the vector backbone sequences.

pINDIGO BACs (Danio Key Library Backbone)

Template: pCRBTIIATI

Primers:

ATII5' (ATI for pINDIGO)

5' - GGG GTT CGC GTC AGC GGG TGT TGG CGG GTG TCG GGG CTG GGC TCG AGC GGC CGC CAG TGT GAT GG – 3'

ATII3'

5' - CCC TAT AGT GAG TCG TAT TAC AAT TCA CTG GCC GTC GTT TAA GCT TGG TAC CGA GCT CGG ATC CAC TAG TAA CGG CCG – 3'

Template: pCRBTIIITK

Primers:

ITKI5' (ITK for pINDIGO)

5' - GCT TGG CGT AAT CAT GGT CAT AGC TGT TTC CTG TGT GAA ACG AGG CGG CCG CAT TAC CC – 3'

ITKI3'

5' - GGG GTT CGC GTT GGC CGA TTC ATT AAT GCA GCT GGC ACG ACC GAG CTC GGA TCC GAA CAA ACG ACC C – 3'

pTARBAC BACs (CHORI-211 and CHORI-73 Backbone)

Template: pCRBTIIATI
Primers:
ATIT5' (ATI for pTARBAC)
5' - GGG CAT CGG TCG AGC TTG ACA TTG TAG GAC TAT ATT GCT CGC TCG AGC GGC CGC CAG TGT GAT GG – 3'

ATIT3'
don't have a pair for inserting ATI into untrimmed CHORI BACs. The 5' primer (shown directly above) works for trimming though.

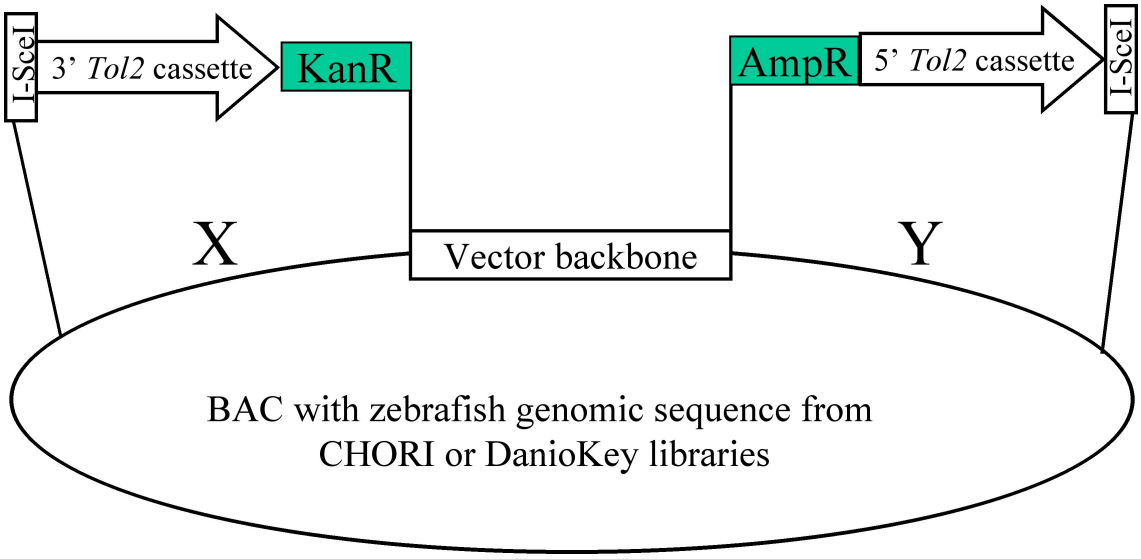
Template: pCRBTIIITK
Primers:
ITKT5' (ITK for pTARBAC)
5' - CCC ACT AGT CAA TTC GGG AGG ATC GAA ACG GCA GAT CGC ACG AGG CGG CCG CAT TAC CC – 3'

ITKT3'
5' - GAT CTG CAG CAT ATC ATG GCG TGT AAT ATG AGA GAC GCC GCC GAG CTC GGA TCC GAA CAA ACG ACC C – 3'

Trimming BAC while simultaneously inserting ATI and ITK

It might be preferable to "trim" a BAC if there are regulatory sequences from an adjacent gene that you might want to exclude. Smaller trimmed BACs also will likely have a higher efficiency of germ-line transmission.

If you would like to trim the BAC (see diagram), you will need to know the orientation of the insert and design a new 3' ATI primer and a new 5' ITK primer. For this, choose 40bp in a region where you want the BAC trimmed and add them to the template binding sequences (non-underlined) in the 3' ATI and 5' ITK primers from above. For the 3' ATI primer, the 40 bp must be the reverse complement. For the 5' ITK primer, use 40 bp of the top strand sequence. The ATI and ITK cassettes will need to be inserted in two sequential BAC recombineering reactions. Confirm targeting by diagnostic digests.



*X and Y will be removed simultaneously with inserting ATI and ITK

Freezing a glycerol stock

In general, BACs are less stable than plasmids. For the shorter term (weeks to months), we store BAC DNA at -20ºC. For the long term, we store BACs in SW105 cells as glycerol stocks.

Grow the culture overnight in LB (with appropriate antibiotics) at 32ºC. The next morning, transfer 1ml of confluent culture to a screw cap tube (preferably a cryo vial). Add 333µl of a 60% glycerol solution made in water and mix. Store at -80ºC.

BAC Maxi Prep – BAC maxipreps are required to generate enough clean DNA for injections. If you would like to sequence any part of the BAC, maxi preps work much better also.

Grow 200 ml of LB culture (with appropriate antibiotics) overnight.

Before starting

As necessary, freeze glycerol stock for long-term storage.
Place ~20 ml elution buffer to 65ºC water bath.
Place a filter (Nucleobond Folded Filters, Cat. # 740 561) into a funnel and place funnel into 250 ml Erlenmeyer flask or equivalent collection vessel.

1. Pellet bacteria
2. Resuspend pellet thoroughly in 50 ml of Qiagen Buffer P1 (50 mM Tris·Cl, pH 8.0, 10 mM EDTA, 100 μg/ml RNase A) using a 10ml serological pipette and pipetting up and down.
3. Add 50 ml Qiagen Buffer P2 [200 mM NaOH, 1% SDS (w/v)], gently invert the tube a couple of times to mix. Let sit at RT for ~5 min.
4. Add 50 ml Qiagen P3 Buffer (3.0 M potassium acetate, pH 5.5),, gently invert the tube a couple of times, white flocculent material will be visible.
5. Pour into funnel with the filter paper. You will no be able to apply the entire volume at one time.
6. Add 10 ml of equilibration buffer (QBT) to the HiSpeed Maxi Tip.
7. After the tip is equilibrated, pour flow through from filter onto tip.
8. After DNA is loaded on the column, add 50 ml wash buffer (QC).
9. After wash flows through, tap the tip on paper towel to remove residual wash.
10. Elute with 5 X 3ml pre-warmed elution buffer (EF). Add 3ml, let flow through, repeat 4 times while keeping the EF in the 65ºC bath.
11. Add 10.5 ml 100% isopropanol. Cool to -20ºC.
12. Spin in Corex Tube at 15,000g for 30' at 4ºC.
13. Pour off supernatant.
14. Wash with 10 ml 70% EtOH.
15. Pour off supernatant. Quick-spin to collect residual wash to the bottom of the tube and remove with a p1000. The pellet may or may not be visible ("splayed up one side of the tube") at this point. Let the pellet air-dry. The pellet will become (more) visible as the wash dries from the Corex tube. Mark the side of the tube containing the pellet with a piece of tape because the pellet will become less visible as it fully dries.
16. Resuspend pellet in 200μl H_20 by gently pipetting the H_20 up the side of the tube and letting it fall to the bottom of the tube. If the pellet looks substantial, you can use 400μl.
17. Transfer the resuspended DNA to an eppendorf tube. Microfuge the resuspended DNA to remove insoluble material. Transfer to a screw cap tube. DNA can be stored at -20ºC.

Plasmid templates

FKF=Frt Kan Frt

Colors
pPCR+AmCyanFKF
pPCR+GFPFKF
pPCR+KaedeFKF
pPCR+ZsYellowFKF
pPCR+ZsYellow-nucFKF
pPCR+TagRFPFKF
pPCR+dsRedExpressFKF
pPCR+dsRedExpress-nucFKF
pPCR+mKateFKF

Inducible Cre alone
pTol2-her4-ERt2CreERt2FKF*
* there is a promoter in this template but the plasmid can be used to amplify ERt2CreERt2FKF as a targeting cassette

Colors plus constitutive and inducible Cres
pBluSKPTagRFP2AcreFKF
pBluSKPmKate2AcreFKF
pCRBTIImKate2AERt2CreERt2FKF

Switch reporter
pPCR+loxPAmCyanSTOPloxPZsYellowFKF

BAC Recombineering Checklist (July, 2011)

Date:
BAC:
Insertion:
Primers:

Preparation of BAC

New BAC
□ - mini-prep BAC and cut with SpeI to confirm identity
□ - transform SW105 cells with BAC, select on LB chl, and identify colony with BAC by mini-prep and digest.

From previous recombineering or glycerol stock
□ - previously ID'ed, already in SW105

<u>BAC recombineering</u>

Preparation of targeting construct by PCR
□ - Set up PCR reaction with minimal template
□ - run 1% gel, isolate product, purify with 4 qiaquick column(s)
□ - treat with DpnI in 200μl digest
□ - purify digest with with minelute column, spin until no wash comes off column, elute with 12μl H_20; Nanodrop 2 μl; concentration____

Preparation of cells
□ - setup 5 ml O/N LB + proper antibiotics culture, shake at 32°C.

Next day,
□ - from O/N culture, start 25ml culture with OD_{600}=.15-.20. Shake at 32°C
□ - turn on refrigerated centrifuge and hot water bath (42°C).
□ - when OD=0.55-0.6, induce half of culture for 15' at 42°C
□ - "fast cool" centrifuge
□ - transform cells keeping them as cold as possible at all times
□ - how much DNA will be transformed into each tube?_____
□ - record Tc following electroporation. UI Tc= ; I Tc=
□ - add 1ml SOC media and let recover shaking 60' at 32°C, plate all on LB_____
□ - incubate O/N in 32°C incubator

Antibiotic Concentrations

We generally make 1000X stock concentrations. Chloramphenicol is made in EtOH. Amp and Kan are made in H_20,

Final concentrations used:
Chloramphenicol, 12.5 μg/ml
Ampicillin, 50 μg/ml
Kanamycin, 25 μg/ml

Example of finding *nkx2.5* containing BACs through Sanger Center Blast Server

As an example, let's search for a BAC containing the nkx2.5 locus and surrounding genomic regions. The full length nkx2.5 cDNA sequence was found here http://www.ncbi.nlm.nih.gov/nuccore/NM_131421. Blasting this sequence retrieves the following real hits:

CR812847.5
[EMBL:CR812847.5]

With the 97% identity between the query and BAC, I know that this BAC contains nkx2.5. I also learn that the 5' end of the locus is towards the left in the graphic because, in the alignment the nucleotide number is increase from left to right for both the query and subject (i.e. BAC).

From the graphic, I learn that this BAC contains ~37.5Kb upstream and ~190Kb downstream of the nkx2.5 locus.

Clicking the EMBL link, I learn that this is BAC contains
Zebrafish DNA sequence from clone DKEY-9I15 in linkage group 14.

The BAC identifier DKEY-9I15 tells me that the BAC is found in the Danio Key library on plate 9, well I 15. It can be ordered here:

zC202J16.00001
[EMBL:CR846079]

With the 97% identity between the query and BAC, I know that this BAC contains nkx2.5. I also learn that the 5' end of the locus is towards the left in the graphic because, in the alignment, the nucleotide number is increase from left to right for both the query and subject (i.e. BAC).

From the graphic, I learn that this BAC contains ~27.5Kb upstream and 2-3Kb downstream.

Clicking the EMBL link, I learn that this is BAC contains
Zebrafish DNA sequence *** SEQUENCING IN PROGRESS *** from clone CH211-202J16

The BAC identifier CH211-202J16 tells me that the BAC is found in the CHORI211 library and resides in plate 202, well J16.

Nonetheless, I would avoid this BAC because it appears to not be fully sequenced.

CU019640.4
[EMBL:CU019640.4]

The alignment, with identity at 97%, tells me this BAC contains the nkx2.5 locus. I also learn that the 5' end of the locus is towards the right in the graphic because the query nucleotide number decreases as the subject (i.e. BAC) number increases. Basically, the nkx2.5 locus is on the complementary strand pointing in the reverse direction.

From the graphic, I learn that this BAC contains ~19Kb upstream and ~23Kb downstream.

Clicking the EMBL link, I learn that this is BAC contains
Zebrafish DNA sequence from clone CH73-269A20 in linkage group 14.

The BAC identifier, tells me that the library is the CHORI 73 and the BAC is located on plate 269 in well A20. This BAC can be ordered here.

>zC194H23.00311
This BAC does not contain nkx2.5 because the identity is quite a bit lower at 74%.

References

Suster, M. L., Abe, G., Schouw, A. and Kawakami, K. (2011). Transposon-mediated BAC transgenesis in zebrafish. *Nat Protoc* 6, 1998-2021.

Warming, S., Costantino, N., Court, D. L., Jenkins, N. A. and Copeland, N. G. (2005). Simple and highly efficient BAC recombineering using galK selection. *Nucleic Acids Res* 33, e36.

Zhou, Y., Cashman, T. J., Nevis, K. R., Obregon, P., Carney, S. A., Liu, Y., Gu, A., Mosimann, C., Sondalle, S., Peterson, R. E. et al. (2011). Latent TGF-beta binding protein 3 identifies a second heart field in zebrafish. *Nature* 474, 645-8.