**FLUORESCENT IN SITU HYBRIDIZATION of CHICKEN BAC-clone DNA to CHICKEN LAMPBRUSH CHROMOSOMES**

**(DNA/DNA+RNA hybridization protocol)**

Chicken BAC DNA clones can be ordered from BAC libraries constructed at the Wageningen Agricultural University (<http://www.lifesciences.sourcebioscience.com/clone-products/non-mammalian/chicken/genomic-chicken-bac-library/>) or at the Children's Hospital Oakland Research Institute (CHORI-261 BAC library, <https://bacpac.chori.org/library.php?id=120>).

1. *BAC DNA isolation using rapid alkaline lysis miniprep method*
2. Inoculate a single isolated bacterial colone into 4-5 ml LB media supplemented with chloramphenicol (20 μg/ul). It is convenient to use a 13-14 ml round bottom polypropylene tube with a snap cap and inoculate bacteria using sterile toothpick. Grow overnight (15-20 hours) shaking at 200 rpm at 37°C.
3. Centrifuge bacterial suspension at 700 g for 7-10 min in a centrifuge equipped with an angle rotor.
4. Discard supernatant. Freeze pellet at -20°C for 30 min.
5. Resuspend pellet in 100 μl ice-cold solution I (10mM EDTA), add 200 μl of solution II (0.2 N NaOH, 1% SDS), mix by inverting several times and leave on ice for 5 min. The content should become almost transparent.
6. Add 150 μl of solution III (3M sodium acetate, pH 5.0) and gently shake. Place on ice for at least 5 min. A thick white E.coli DNA and protein flakelike precipitate should form.
7. Centrifuge at 13,000 g for 10 min.
8. Transfer supernatant to a 1.5ml Eppendorf tube avoiding any white precipitated material.
9. Add 450 μl 5M LiCl, vortex and put on ice for 30 minutes.
10. Centrifuge at the full speed (13,000 g) for 10 minutes.
11. Transfer supernatant to a new 1.5 ml Eppendorf tube.
12. Add 0.7-1 volume of isopropanol or 2.5-3 volumes of ice-cold ethanol. Mix by inverting and place on ice for at least 5 min. At this stage samples can be frozen at -20°C overnight.
13. Centrifuge at 13,000 g for 15 min at +4°C.
14. Remove supernatant and rinse the pellet with 200 μl of 70% ethanol by inverting the tube.
15. Centrifuge at 13,000 g for 15 min at +4°C.
16. Carefully remove as much supernatant as possible. It should be remembered that at this step pellet dislodges easily from tube bottom.
17. Air dry pellet at room temperature. When most of the ethanol evaporates, the DNA pellet should turn from white to transparent.
18. Dissolve the pellet in 20 μl of water or TE, pH 8.0.
19. Add RNase A to final concentration 100-150 μg/ml and incubate for 1-2 hours at 37°C.
20. Precipitate DNA adding 5M sodium chloride to final concentration of 0.3M and 2.5 volumes of ice-cold ethanol as described in steps I.12-I.17. Store purified BAC DNA at

-20°С.

*Solutions:*

Solution I: 10mM EDTA

Solution II: 0.2 N NaOH, 1% SDS

Solution III: 3M sodium acetate, pH 5.0

TE buffer: 10 mM Tris, pH 8.0; 1 mM EDTA

1. *Amplification* *of BAC DNA with DOP-PCR*
2. Prepare DOP-PCR mixture:

|  |  |  |
| --- | --- | --- |
|  | Final concentration | μl to add |
| 10X Taq-polymerase PCR buffer (without Mg2+) | 1X | 2 |
| MgCl2 (25 mM) | 2 mM | 1.6 |
| dNTPs mix (2.5mM each of dATP, dGTP, dCTP, dTTP) | 0.2 mM each  | 1.6 |
| 6MW primer (10 μM) | 0.2 μM | 0.4 |
| Isolated BAC-clone DNA | Approx. 50-100 ng | to be calculated |
| Taq-polymerase (5u/μl) | 2.5 u | 0.5 |
| H2O  |  | up to 20 μl |

1. Mix thoroughly, centrifuge and place the tubes in thermocycler
2. Perform DOP-PCR cycles as follows

Initial denaturation at 94ºС for 5 min

5 cycles of 94ºС for 1 min, 30ºС for 1.5 min and 72ºС for 3 min

25 cycles of 94ºС for 1 min, 55ºС for 1 min, 72ºС for 1.5 min

Final extension step of 72ºС for 10 min

1. Check PCR products by electrophoresis in a 1% agarose gel. A smear of bands from 200 to 2000 bp should be visible (fig.1).



**Figure 1.** Amplified DNA of chicken BAC-clones using DOP-PCR visualized in 1% agarose gel stained by ethidium bromide. 72A10, 15N12, 114G22, 169K18, 28L10 – names of BAC-clones from CHORI-261 BAC-library, K- - negative control DOP-PCR amplification (without template DNA), M – DNA fragment size marker, bp.

1. *Probe labeling with digoxigenin or biotin*
2. Prepare biotin or digoxigenin DOP-PCR mixture

|  |  |  |
| --- | --- | --- |
|  | Final concentration | μl to add |
| 10X Taq-polymerase PCR buffer (without Mg2+) | 1X | 2 |
| MgCl2 (25 mM) | 2 mM | 1.6 |
| dNTPs ‘hot’-mix (2 mM each of dATP, dGTP, dCTP and 1.6 mM dTTP) | 0.2 mM dATP, dGTP, dCTP and 0.16 mM dTTP  | 2 |
| Biotin-dUTP or digoxigenin-dUTP (1mM) | 0,04 мМ | 0.7 |
| 6MW primer (10 μM) | 0.2 μM | 0.4 |
| DOP-PCR amplified BAC-clone DNA | Approx. 50-100 ng | 1 |
| Taq-polymerase (5u/ μl) | 2.5 u | 0.5 |
| H2O  |  | up to 20 μl |

1. Mix thoroughly, centrifuge and place the tubes in thermocycler
2. Perform 35 DOP-PCR cycles as follows

Initial denaturation at 94ºС for 5 min

35 cycles of 94ºС for 1 min, 55ºС for 1 min and 72ºС for 1.5 min

Final extension step of 72ºС for 10 min

1. Check PCR products by electrophoresis in a 1% agarose gel. A smear of bands from 200 to 1000 bp should be visible.
2. Add to labeled DNA 50X excess of sheared salmon sperm DNA. Precipitate mix of BAC-clone and salmon sperm DNA with ice-cold ethanol, rinse with 70% EtOH, air-dry pellet as described in steps I.12-I.16.
3. Dissolve precipitated DNA in hybridization mixture (50% formamide, 2XSSC, 10% dextran sulphate) to final concentration of labeled DNA of 15-20 ng/μl. It is recommended to start reconstitution of the precipitated DNA with pellet dissolving in a corresponding volume of 100% formamide by vigorous vortexing for 1 hour at room temperature. Then add a required volume of ‘master mix’ (20% dextran sulphate, 4XSSC). Labelled DNA in hybridization mixture can be stored frozen for several years.

Example of preparing of probe hybridization mixture for FISH on chicken LBCs

|  |  |  |  |
| --- | --- | --- | --- |
|  | Final concentration in hybridization mixture | μl to add | What to do |
| Biotin or digoxigenin labeled BAC-clone DNA, 200 ng/μl | 20 ng/μl | 1 | Mix, precipitate and air-dry pellet |
| Sheared salmon sperm DNA (50X excess), 10 mg/ml  | 1 μg/μl | 1 |
| Formamide (100%) | 50%  | 5 | Add in the first place, vortex vigorously |
| Master mix (20% dextran sulphate, 4XSSC) | 10% dextran sulphate, 2XSSC | 5 | Add to DNA dissolved in formamide |

*Solutions:*

Hybridization mixture: 50% formamide, 10% dextran sulphate, 2XSSC

Master mix: 20% dextran sulphate, 4XSSC

1. *Fluorescent in situ hybridization*

For FISH dry the lampbrush chromosome preparations after fixation and store them dried at room temperature in slide boxes. They can be stored up a year. Before using examine slides under the phase contrast microscope.

***Day 1***

No RNase and protease treatments.

Dehydratation after storage

1. Dehydrate slides with lampbrush chromosome sets in 70% and 96% ethanol washes (5 min per wash).
2. Allow slides to air dry at room temperature.

Denaturation:

1. Prewarm 50 ml of denaturing solution (70% formamide, 2X SSC) to 70-72°C in a Coplin jar. Using a calibrated thermometer, check the temperature of the solution inside the jar before adding slides.
2. Incubate slides for 3 minutes in the preheated denaturing solution with slight agitation. It is recommended do not denature more than two-three slides at the time in the same jar because each room temperature slide decreases the temperature of denaturing solution by 1°C. Allow denaturing solution to reheat to 70°C before placing other slides. The denaturation time of lampbrush chromosomes should not exceed 3 min!
3. Immediately immerge slides into cold (-20°C) 70% ethanol, followed by cold 80% and cold 96% ethanol washes in Coplin jars or in 50ml Falcon tubes (3 min per wash).
4. Air dry slides at room temperature.

Probe denaturation:

1. Aliquot required quantity of prepared probe hybridization mixture in a new 0.5 ml Eppendorf tube, roll with parafilm, and denature probe by boiling for 5 minutes in water bath then quick put it on ice for 5 minutes.
2. Add 7 μl of denatured probe to each lampbrush slide with marked well chambers (see ‘Methods for making lampbrush chromosome preparations from oocytes of birds’, http://projects.exeter.ac.uk/lampbrush/protocols.htm), cover with a 18x18 mm coverslip (make sure that air bubbles are absent) and seal with rubber cement.
3. Incubate slides overnight (at least 16 hours) in a humid chamber at 37°C.
4. Denaturation of chromosomal and probe DNA can be performed simultaneously, in a thermocycler equipped with a slide-block or in a heating slide dry-block. For this, add 7 μl of a probe in hybridization mixture to lampbrush slide, cover with a 18x18 mm coverslip and seal with rubber cement. Denature simultaneously at 82°C for 5 min, slowly cool down to 37°C. Incubate slides overnight (at least 16 hours) in a humid chamber at 37°C.

*Solutions:*

Denaturing solution: 70% formamide, 2X SSC

20XSSC: 3 M sodium chloride, 0.3 M trisodium citrate; adjust to pH 7.0 with HCl.

***Day 2***

Post-hybridization washes and detection:

1. Prewarm four Coplin jars with 0.2XSSC (two jars) and 2XSSC (two jars) to 62°C.
2. Remove slides from humid chamber, take away rubber cement and place slides in a Сoplin jar with 2XSSC at room temperature until coverslips peel away.
3. Transfer slides to the first Сoplin jar with 0.2X SSC at 62°C and incubate for 5 minutes with agitation. Transfer slides to the second coplin jar with 0.2XSSC and that in 2XSSC containing jars and incubate for 5 min in each wash.
4. Transfer slides to a Coplin jar containing room temperature 4XSSC+0.1Tween-20.
5. DO NOT ALLOW SLIDES TO DRY AT ANY TIME AFTER THIS STEP!
6. Take out one slide at a time from 4XSSC+0.1% Tween-20, quickly absorb excess liquid from the slide using a paper towel. Immediately apply 50 μl of blocking solution (1% blocking reagent in 4XSSC+0.1% Tween-20 or 3% BSA in 4XSSC+0.1% Tween-20), cover with a piece of parafilm and incubate in a humid chamber at 37°C for 35-40 minutes.
7. After blocking, remove parafilm and absorb excess blocking liquid from the slide using a paper towel. Add 50 μl of blocking solution containing mouse anti-DIG antibody and/or avidin-Alexa488. Incubate 35-40 minutes at 37°C in a humid chamber.
8. Remove slides from a humid chamber and wash them in three changes of 4XSSC+0.1% Tween-20 prewarmed to 37°C (5 minutes per wash, with agitation).
9. Take out one slide at a time from 4XSSC+0.1% Tween-20, quickly absorb excess liquid from the slide using a paper towel. Immediately apply 50 μl of blocking solution containing anti-mouse Cy3 antibody and/or biotinilated anti-avidin. Cover with parafilm and incubate 35-40 minutes at 37°C in a humid chamber.
10. Repeat 4XSSC+0.1%Tween-20 washes as described in step 8.
11. Take out one slide at a time from 4XSSC+0.1% Tween-20, quickly absorb excess liquid from the slide using a paper towel. Immediately apply 50 μl of blocking solution containing avidin-Alexa488. Cover with parafilm and incubate 35-40 minutes at 37°C in a humid chamber.
12. Repeat 4XSSC+0.1%Tween-20 washes as described in step 8.
13. Rinse slides one by one in a glass beaker containing room temperature 2XSSC, dehydrate them in 70% and 96% ethanol for 5 minutes each and air dry.
14. Add 7 μl of LBC antifade solution containing DAPI to each slide (prewarm to ambient temperature before adding since this solution is very viscous at -20°C) and cover with a coverslip avoiding any bubbles by pressing coverslip gently with the forceps or pencil.
15. Examine using fluorescence microscope. Slides can be stored at +4°C.

*Solutions:*

20XSSC: 3 M sodium chloride, 0.3 M trisodium citrate; adjust to pH 7.0 with HCl.

Blocking solution: 1% blocking reagent (Roche) in 4XSSC+0.1% Tween-20.

LBC antifade solution with DAPI: 50% non-fluorescent glycerine, 1-1.2% DABCO, 2XSSC, 50 ng/ml DAPI.

*References*

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Chicken NOR-bearing chromosome 16 at the lampbrush stage identified using DNA/DNA+RNA FISH with 18S-5.8S-28S rRNA genes containing BAC-clone WAG137G04 from the Wageningen BAC-library. The BAC-clone DNA was labeled with biotin-16-dUTP (Sileks, Russia), lampbrush chromosome was counterstained with DAPI. Bar = 5 μm.