C9ORF72 DIG probe Southern Blot Protocol

Notes:

STOP = a point in the protocol where you can stop/store samples. Otherwise, proceed through until the next STOP to ensure that samples/gel/membrane are/is not compromised

Step 1 - Prepare Genomic Tail DNA

Materials

- QIAGEN DNeasy Blood & Tissue Kit (QIAGEN, cat no.69504 or 69506).
- 100% EtOH
- Ultra-pure distilled water

➢ Before starting, place Buffer AL and Buffer ATL at 56°C to ensure that salts are dissolved. Use the oven/incubator in 1160 for this step.

Procedure

☐ Sample 0.5cm of tail into a 1.5mL tube (gDNA extraction works better on tail tips from mice <4 weeks of age)

Tips:

Tail length should not be less than 0.5 cm. Reason: not enough DNA yield if < 0.5cm (DNA concentration should be above 85ng/µL).

☐ Make a mixture of **180µL PREWARMED Buffer ATL** and **20µL proteinase K** for each tail and add 200µL. Make sure that ATL is mixed by swirling it prior to adding.

Tips:

Make sure that the tail tip is at the bottom of the tube, submerged in the lysis buffer.

☐ Incubate at 56°C until completely lysed. Vortex occasionally during incubation.

Tips:

Tail lysis can be completed in 2 hours, especially if you vortex the tail every 30 minutes.
DO NOT INCUBATE OVERNIGHT
During the digest is a great time to label your columns and elution tubes!

☐ **Vortex 15 sec** directly before adding the **PREWARMED** Buffer AL + 100% Ethanol
☐ Add **400uL PREWARMED Buffer AL + 100% Ethanol** combined in equal parts (1:1).
☐ Mix thoroughly by pulse-vortexing for 5-10 s
☐ Pipet 700 uL of the mixture into a DNeasy Mini spin column in a 2mL collection tube.
☐ Centrifuge at **≥ 6000 x g (8000rpm) for 1 min**. Discard the flow-through and collection tube.

- ☐ Place the spin column in a new 2mL collection tube. Add **500uL Buffer AW1**.
- ☐ Centrifuge for **1min** at **≥ 6000 x g/8000 rpm**. Discard the flow-through & collection tube.
- ☐ Place the spin column in a new 2mL collection tube. Add **500uL Buffer AW2**.
- ☐ Centrifuge for **3min** at **≥ 20,000 x g** (14,000rpm). Discard the flow-through and collection tube.
- ☐ To avoid buffer contamination (230/260 ratio > 2.3 or < 1.8) and to dry the column, re-centrifuge at 14,000 rpm for 1 min.
- ☐ Transfer the spin column to a labeled 1.5mL tube. Elute the DNA by adding 50uL of Tris-EDTA pH 8.0 to the center of the column. Incubate for 5 min at RT. Centrifuge for **1min at ≥ 6000 x g**.
- ☐ Perform a second elution by adding another 50uL of Tris-EDTA pH 8.0 to the center of the column. Incubate for 5 min at RT. Centrifuge for **1min at ≥ 6000 x g**.
- ☐ Measure concentration of the DNA using the Nano-drop.
- ☐ Store DNA at 4°C.

STOP, or proceed to DNA Digestion with XbaI

Step 2 - Genomic DNA Digestion

Materials

- XbaI (New England Biolabs, cat no. R0145L) at 20U/µL
  - KEEP ON ICE OR IN ENZYME COOLER
- 10X CutSmart buffer (New England Biolabs, cat no.B7204S)
- Ultra-pure distilled water

Procedure

Digest 2 µg or the desired amount of genomic DNA with XbaI in a **final volume of 29uL**:

- ☐ Make a mixture of enzyme and buffer: **1 µL** of XbaI, **2.9µL** 10X CutSmart buffer, and **1.1µL** water per digest.
- ☐ Add **5µL** of **enzyme/buffer mixture** to labeled 1.5mL Eppendorf tubes (Req, sample ID, JR).
- ☐ Add the amount of gDNA/BAC DNA desired in **µl [amount of DNA desired (ng) /DNA concentration ng/µL]** and **DI water** to make a total volume of **29µL**.
- ☐ Incubate XbaI digests in oven overnight at 37°C.
- ☐ In the morning, add 0.75µL of XbaI, mix, and continue at 37°C for 3 hours or more. Either proceed to loading the digests on a gel or store digests at -20°C.

Take the blocking reagent out of freezer to let it thaw.

STOP, Or proceed to load the gel(s).

Step 3 - Gel electrophoresis separation

Materials

- Gel Box
- Gel Tray
- Gasket
- 32 well x 1.5mm comb
- A tape
- Gel buffer: 50X TAE stock [Fisher Scientific, cat#BP13324]
- Agarose: [SeaKem LE Agarose (Lonza, cat#50005)]
- Ethidium Bromide
- GelPilot 1kb ladder [9 fragments: 1000–10,000 bp, QIAGEN, cat#239085]
- DNA Molecular Weight Marker VII, DIG-labeled [Sigma, cat# 11669940910]
- DNA Gel Loading Dye (6X) [ThermoFisher Scientific, cat# R0611]
- 96 well PCR plate [1402-9598 TempPlate 96-well No-Skirt 0.2mL CPR plate]
- *XbaI* digested DNA samples
- 8-Channel Electronic Pipette [INTEGRA VIAFLO II 8-Channel, 0-50µl, cat#4626]

Procedure

1- Gel preparation

- ☐ Prepare **3L** of **1X TAE**: Mix **60mL 50X TAE** + **2940mL DI water** for each GEL
- ☐ Prepare **0.7% agarose gel**: add **3.5g agarose** to **500mL 1X TAE** in 1L bottle
- ☐ Microwave on **high** approx. **4-6min** [leave the cap loose]
- ☐ Swirl and check that the agarose is completely melted
- ☐ Let the agarose cool on your bench until touching the bottom of the beaker with your bare hand doesn't burn you and **add 25µl** of **ethidium bromide**
- ☐ Tape the ends and place rubber gasketed end pieces in a large gel tray to seal (21x27cm)
- ☐ Place the sealed tray into the gel box and level if necessary
- ☐ Poor the cooled agarose solution into the gel tray and place a **32well X 1.5mm comb** into the slot nearest the end of the tray
- ☐ Let the agarose solidify at least 30 minutes
- ☐ Remove tape, gaskets and the comb and fill the gel box with 1 x TAE until the gel is just submerged.

2- Load and run the gel

The DIG-labeled and Gel Pilot markers are found in the "chocolate" fridge in 1130

- ☐ Prepare 30uL of DIG-Labeled marker VII 0.**1ng/µL (**for 2 membranes):

3uL of 10x Smart buffer, **0.3ul** of 10ug/ul DIG-labeled marker VII, **21.7** of H2O, **5ul** of 6X loading dye

- Load 15 **µl** of **DIG-labeled marker VII per membrane** on the first well of the gel
- Load **15 µl** of **GelPilot 1kb ladder** into the **last well** of the gel
- Load **38 µl** of XbaI digested DNA **(30µl XbaI digest** + **8µl 6X loading dye**) into the wells using INTEGRA VIAFLO II 8-Channel, 0-50µl **[**tips spacing config: 4.5mm – 9mm – 6.3mm]
- Run the gel at **70 mV** for **16h** (set power supply to **70mV** and the time to **960minutes**).

Step 4 - Capillary Transfer

***NOTE*:** The gel is a low-agarose percentage gel, so it is **VERY** fragile. Please be mindful of this when photographing and setting up the transfer—the gel should stay in the gel tray or be supported by plates or spatulas at all times to ensure that the gel stays intact.

Preparation

The next morning the xylene cyanol dye should be at ~ 9 cm and bromophenol blue dye at ~ 20.5 cm from the top (6X loading dye components)

- Capture a picture of the gel with a ruler over the Gel Pilot marker in the last lane using the trans-illuminator [exposure time: 80 ms].
- Save the image in Z:\Southerns Genotyping\Southern Blot\On-going southern
- Trim the gel and retain the portion of the gel between **10 and 2kb fragments** of the Gel Pilot marker:

a) Using a razor blade cut across the gel at [16.5-18.5] cm from the top and discard the bottom portion
b) Using the razor cut a small notch on the lower left corner to identify lane 1.
c) Finally cut across the gel at [6.5-8.5] cm from the top and discard the top portion with the wells. The dimension of the gel after trimming is usually 8.5-10cm X 20cm.

- Capture a picture of the gel and save it in Z:\Southerns Genotyping\Southern Blot\On-going southern

Capillary Transfer

Materials

- Whatman Grade 3MM Chr Blotting Paper, sheet, 46 × 57 cm [cat#3030-917]
- GB005 Blotting Paper [GE Healthcare Life Sciences, cat# 10426994; 580 X 580mm]
- Roche positively charged nylon membrane [Sigma-Aldrich, cat#1141724001; 30cm X 3m]
- Cescolite tray [CL810T, 8x10”]
- 20x SSC [Fisher Scientific, cat#BP1325-20]
- UV Crosslinker [Fisher Scientific cat#13-245-221]

- DIG Easy Hybridization Granules [Sigma-Aldrich cat#11796895001]

Procedure

Pre-warm ultra-pure distilled water at 37°C

Do not treat the gel with HCl if DNA fragments are <10kb (omit depurination).

- Transfer the gel into a Cescolite tray (white tray) wrapped with plastic wrap
- Gently shake the gel **2 X 15 min** in **500 mL** of **Denaturation solution 0.5M NaOH, 1.5MNaCl** (**87.66 g NaCl** + **20 g NaOH** bring volume to **1 L with DI water**) prepared fresh at **30rpm** on MaxQ 2000 orbital shaker.
- Discard the denaturation solution and Rinse the gel with **500mL of DI water**.
- Gently shake the gel in **500mL** of **Neutralization solution 0.5M Tris pH7.5, 1.5MNaCl (87.66 g NaCl, 250ml 2M Tris pH 7.5** bring volume to **1L with DI water**) for 30 **min**.
- Prepare the transfer tray and wick:
- Cut Whatman 3MM Chromatography paper 46 X 57cm to 27 X 46cm (gel tray dimensions).
- Wrap the paper over the flat bottom of a large gel tray and place into a Cescolite tray (white tray).
- Fill the tray with about **2L** of **10xSSC** (dilute 20x SSC, 1:1 dilution with DI water).
- Remove air bubbles by gently rolling a pipette over the surface. The paper should be saturated.
- Cut (per membrane, size of trimmed gel):
  - Whatman 3MM chromatography paper into 4 pieces
  - 8 pieces of GB005 Blotting
  - 1 Roche positively charged nylon membrane (Sigma-Aldrich, cat no. 1141724001; 30cm X 3m) to the size of the trimmed gel.
- **Pre-wet the membrane** first briefly in DI water then change to **2XSSC**.
- Invert the gel (wells facing down) onto a tray and then slide off onto the middle of the transfer wick. Gently roll pipette over surface to remove air bubbles under the gel.
- Place the membrane on the gel [membrane facing the flat side of the gel] and gently roll pipette over to remove air bubbles.
- Lay pieces of plastic wrap over the wick to cover the gel sides to create a barrier between the wick and the stack of paper towels. This will maintain capillary flow through the gel.
- Wet 2 pieces of the Whatman in **2XSSC** and layer on the membrane one at a time.
- Layer on the 2 remaining Whatman dry and the 8 Gel Blot dry.
- Layer on a stack of paper towels at least 4 inches thick. Place a weight (no more than 500 g) on the stack of towels. I suggest an unopened box of gloves.
- After 2-3 hours remove wet paper towels and replace with dry ones. Allow to transfer overnight (typically **16h**).
- Incubate pre-warmed nuclease free water at 37°C for pre-hyb step (DIG Easy Hyb buffer preparation).

- Pre-warm ultra-pure distilled water at 37°C, overnight.

The next day take down the transfer:

Note: Avoid touching the DNA side as much as possible during the entire process!

- Discard the paper towels and all the layers of blotting paper. Leave the membrane on top of the gel.
- Cut the corner of the membrane corresponding to the notch at the lower left on the gel to indicate lane 1.
- Carefully lift the membrane from gel and place it on the "lidded incubation tray" filled with 2x SSC. DNA side (side facing gel) facing up!
- Using a soft lead (blue) pencil write the Req numbers/Southern title at the top of the membrane on the DNA side.
- Rinse in 2XSSC 5 min using the shaker set up on low speed: 30rpm. Again, make sure the DNA is still facing up while the membrane is in the tray!
- Lay the membrane flat on a dry piece of Whatman, with the DNA facing up!
- Clamp the membrane one the Whatman paper with paper clips in the corners, DNA facing up, and let it dry on a bench top or in the chemical hood to increase air flow. DO NOT apply Whatman paper on the DNA side to speed up drying. Let dry for at least 15 min.

***NOTE*:** If you apply Whatman on the DNA side to speed up the drying, you're lifting the DNA up the membrane and can ruin the signal. The bond between DNA and membrane is very weak before the cross-liking so **NOTHING** should touch the DNA side of the membrane.

- Use the Whatman paper as a support to move the membrane in the cross linker. UV crosslink in the UV Crosslinker (Fisher Scientific cat no. 13-245-221) with DNA-side facing up. Use "Energy mode" with 120,000µJ.
- Rinse the membrane in 2XSSC for 5 min at low rpm: 30.
- Proceed with pre-hybridization or store at 4°C

  Note: If hybridization is not to follow, store the membrane at 4°C, place membrane on Whatman paper for support, DNA facing up, and wrap it in plastic wrap, avoiding the contact of DNA with any piece of paper.

Preparation of DIG Easy Hyb Buffer:

- Add a small stir bar and 64mL pre-warmed nuclease-free water to the plastic bottle of DIG Easy Hybridization Granules (Sigma-Aldrich cat no. 11796895001).
- Dissolve by stirring at 37°C. Dissolving normally takes 1-2h.
- Pre-heat appropriate volume (pre-hyb + hyb) of DIG Easy Hyb to 48°C in a hybridization oven (VWR/Boekel, cat no. 230402V).

Step 5 - Pre-hybridization and Hybridization

Materials

- DIG Easy Hybridization Granules (Sigma-Aldrich cat no. 11796895001)
- Roller bottle (35X300mm, VWR, cat no. 502-0300R).
- Hybridization Oven

Pre-hybridization

The pre-hybridization step (also known as blocking) is done to minimize non-specific attachments of a probe to the nylon membrane. A blocking agent prevents the probe from sticking to the membrane, ensuring that it will only interact with the desired DNA bands that have been transferred to the membrane.

- Roll the damp membrane DNA side facing in and insert into a large roller bottle (35X300mm, VWR, cat no. 502-0300R).
- Add **2XSSC** to the bottle and gently roll so the membrane will stick to the wall of the bottle. Avoid trapping bubbles between the membrane and the glass. If membrane overlaps itself in the bottle, top the membrane with a nylon mesh (VWR, cat no. 40000-424; 23x23cm) as a spacer.
- Decant the 2XSSC.
- Add **25mL of DIG Easy Hyb** to the roller bottle
- In the Hybridization Oven, place another bottle for balance and set **roller speed** at **5**. Rotate for 3 hours or more at 48°C.
- Keep the DIG Easy Hyb at 48°C.

Hybridization

- Add DIG-labeled oligonucleotide probe (Prepared by IDT, Sequence: $(GGGGCC)_5$ into pre-warmed DIG Easy Hyb to make a 100ng/mL solution. For example: add **2.5µL** into **25mL** DIG Easy Hyb

Note: 5µL aliquots at 1µg/µL of the DIG-labeled probe are stored in -20°C freezer "strawberry".

- Mix well by swirling. Do not vortex and avoid foaming.
- Decant pre-hybridization buffer from roller bottle. Immediately add DIG Hyb mixture onto the wall of the roller bottle (avoid bubbles).
- Rotate with speed of **5** at **48°C** overnight. Try not to probe more than 18 hours.
- Pre-warm overnight 2 flasks 100mL each of 2XSSC, 0.5% SDS buffer, one at 48°C and the second one at 68°C.
- Pre-warm overnight 100mL of 0.5X SSC, 0.5%SDS at 68°C overnight
- Pre-warm overnight 100mL of 0.15X SSC, 0.5%SDS at 68°C overnight

Step 6 - Stringency Washes

Low stringency wash:

- 1st Wash of the membrane with 100mL 2XSSC; 0.5% SDS for 15min, while the oven ramps from 48°C to 68°C. Increase the rolling speed from 5 to 15.
- Decant washing buffer, and wash again in 100mL 2XSSC; 0.5%SDS at 68°C for 15min.

High stringency washes:

- Wash for 15 min in 100mL 0.5× SSC; 0.5% SDS at 68°C.
- Wash for 15 min in 100mL 0.15x SSC; 0.5% SDS at 68°C.

Antibody detection

Preparation of detection solutions:

10X Maleic Acid Buffer: **1M Maleic Acid** (Sigma-Aldrich, cat no. M0375-500G)**, 1.5 M NaCl**; (87.66 g NaCl, 116.07g Maleic Acid bring volume to 1 L with DI water) pH to 7.5 adjust with NaOH pellets –add 10g at a time, up to 70g. Add 1g at a time taking frequent pH measurements. 75-80g of NaOH pellets well be needed to attain the proper pH. If the pH rises above 7.5 the solution can be adjusted downward with 12M HCl. Store at RT (15-25°C) Dilute 10X to 1X to make Blocking and Washing solutions.

Blocking Solution: **10X Blocking Stock**: Dissolve Blocking Reagent (Sigma-Aldrich, cat no. 11096176001) in 1x Maleic Acid buffer to a final concentration of 10% (w/v) with stirring and heating either on hot plate or in a microwave oven. Autoclave the solution. When cooled aliquot and store at -20°C.

1X blocking solution: Dilute 10× Blocking Stock 1:10 with 1X Maleic Acid buffer. Always prepare fresh. Suggest 250mL per membrane - 150mL for block and 100mL for antibody.

- Formula: 25 mL 10X blocking solution + 25 mL 10X Maleic Acid buffer + 200 mL of DI water.

Antibody Solution: Centrifuge Anti-Digoxigenin-AP Fab fragments (Sigma-Aldrich, cat no. 11093274910) for 5 min at 10,000 rpm in the original vial prior to each use

- Pipet carefully from the surface.
- Dilute 1:10000 (75 mU/mL final). Suggest making 100mL antibody solution per membrane.
- Formula: 10µL antibody + 100mL 1X blocking solution.

Washing buffer: 0.1 M Maleic acid, 0.15 M NaCl; pH 7.5; 0.3% (v/v) Tween 20. Store at RT.

- Suggest 150mL washes X 4 = 600mL total for each membrane. Add 1.8mL Tween 20 to 600mL of 1X Maleic Acid buffer and stir to mix.
- Formula: 60mL 10X Maleic acid + 540mL of DI water + 1.8 mL Tween 20.

Detection Buffer: 0.1 M Tris-HCl, 0.1 M NaCl, pH **9.5** (20°C). Store in plastic container at RT. pH is critical.

- Suggest 150mL washes X 2 =300mL total for each membrane.
- Formula: 30mL of 10X detection buffer + 270 mL DI water

Detection procedure

- After stringency washes, transfer the membrane from the roller bottle into a plastic tray Perfect Western (Genhunter, cat no. B111 or B112, 20.7x15.9.)
- Rinse the membrane for 5 min in **Washing Buffer** on shaker at RT, speed of 115RPM. Use enough buffer (~150mL) to submerge.
- Discard washing buffer and block in 150mL **Blocking Solution** gently shaking at low speed on orbital shaker 30rpm. The blocking can go from 30min up to 2 hours.
- Discard Blocking Solution. Add 100mL **Antibody Solution**.
- Incubate with gentle shaking for 30 min at RT.
- Discard the Antibody Solution. Wash membrane 3 X 15 min in 150 mL **Washing Buffer** on a shaker with vigorous shaking, speed of 120 rpm.
- Briefly rinse membrane with 150mL **Detection Buffer**. Wash membrane 5 min in 150 mL fresh **Detection Buffer**.

Sealing the membrane

- Cut open on the 2 long edges of a sheet protector (Universal Top loading sheet protector, standard gauge, clear, letter size #UNV21124).
- Dry the membrane using Whatman paper and place DNA side up in the sheet protector
- Apply around 1 mL (30 drops which will cover the whole membrane) of **Ready-to-use CSPD** (Sigma-Aldrich, cat no. 11755633001) drop-wise over the surface of the membrane.
- Immediately slowly roll the sheet protector down trying not to trap bubbles.
- Gently roll over the surface with a pipet from the sealed edge to the other side until the CSPD is spread evenly over the membrane.
- Do not let air bubbles form between the membrane and the upper surface of the sheet protector. Incubate for **5 min at RT.**
- Squeeze air bubbles and excess liquid from the sealed side and seal the edges of the sheet protector.
- When using CSPD, incubate membrane wrapped in foil to protect from light for **10 min at 37°C.** This enhances the chemiluminescence reaction.
- Place the sealed membrane in an X-ray cassette. Expose the membrane at RT (DNA side up) to X-ray film (BIOMAX Light Film Standard Chemiluminescent 8X10 in Sigma-

Aldrich, cat no. 178-8207) over the weekend. May only require an overnight exposure—it depends on the quality of the DNA, etc.

DAY6 – Film development

- Turn on the X-ray film processor and wait for the ready light to come on (takes around 20min). Feed in 1 used films before feeding in the film.

  Scanning the films

  With the EPSON Scan software scan the film right side facing down using the Epson Perfection V800 photo scanner.
- Take out the white board, so there are 2 glass surfaces.

Scanner settings: Document type: Film (with Film area guide). Film type: positive film. Image type: 24 bit color. Resolution: 300dpi. Document size: W8.00 H 10.00. Target size: original. Take a Preview scan and adjust the image by rotating. Scan. Save image in TIFF format. Place image into the PowerPoint record of the Southern.