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Standard Operating Procedures	
Subject: NAPPA	Effective: 7/1/09
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Protocol: DNA prep

Material/Equipment	**Amount for one 96-well block**
TB culture medium (KPI+Ampicillin)	1.5 mL
96-pin device (Boekel 140500)	1
Solution 1	200 uL/well
Solution 2	200 uL/well
Solution 3	200 uL/well
Isopropanol	600 uL/well
Solution N2	200 uL/well
Solution N3	2000 uL/well
Solution N5	300 uL/well
800 uL glass fiber MBPP 25 micron filter plate (Whatman 13503-040)	1
Deep-well block	2
Gas permeable plate seal	1
Aluminum plate seal	2
ATR Multitron shaker (37°C)	1
Centrifuge, Eppendorf 5810	1
Eppendorf Thermomixer	1
Omni plate (Nunc 242811)	
LB	
Agar	
Beckman Allegra X15R centrifuge	
Beckman Avanti JE centrifuge	
350 uL 96-well plate (Greiner 651201) for alkaline lysis DNA prep	
800 uL 96-well block (Abgene AB-0859) for Nucleobond prep	
Nucleobond resin (Machery-Nagel custom order)	
Resin addition setup including modified PCR plate	
Thermo-Fisher WellMate Bulk Liquid Dispenser	
Vacuum manifold with liquid waste trap	
Beckman Biomek FX 96/SPAN8 Liquid Handler	

1) If necessary, make up the ampicillin stock: 1 g ampicillin in a total of 10 mL water. Store at -20°C.

2) **Prepare LB/Agar omni plate**. To prepare LB/Agar, follow the "Pouring LB Agar Plates" protocol. Pour 30-40 mL on to each omni plate. Allow plates to cool at room temperature until LB/Agar solidifies. Wrap the plates, in groups of five or fewer, in saran wrap and cover with aluminum foil before storing, upside-down, at 4°C until needed.

3) **Pre-dry LB/Agar plates under laminar-flow hood** for approximately 15 mins. before spotting diluted glycerol stock. Do not over-dry the plates. Make sure that the glycerol stock dilutions are done in a timely fashion.

4) **Spot glycerol stock on LB/Agar plate**. Dilute glycerol stock in LB (1:300, usually 2 uL in 600 uL of LB). This dilution can be done on the Biomek FX. Shake for 10 mins. Spot 3 uL of the diluted stock onto the LB/Agar plate using the Biomek FX. Allow plate to dry under laminar flow hood until completely dry. Do not over-dry the plates. Incubate in 37°C oven, upside-down, overnight. This incubation step should last approximately 16-18 hrs; however, discretion should be used to judge colony growth. Optimally, each spot should contain abutting, but not overly grown colonies.

5) **Inoculate culture**. Prepare a deep-well block for the cultures, 1.5 mL per well of medium: TB and 100 ug/mL ampicillin stock solution.

6) **Sterilize the 96-pin device (replicator)** in 80% ethanol, then flame. Let the pins cool.

7) Using the 96-pin device, **inoculate the culture block from the agar plate**. Be sure to flame the pins thoroughly between inoculations even if it is from the same agar plate. Remember to let the pins cool after each flaming. Approximately 6 blocks can be inoculated from single agar plate.

8) **Cover the block with a gas permeable seal and put it on a shaker for 24 hours** at 37°C, 300 rpm or 800 rpm depending on shaker. The ATR Multitron shaker, set to shake at 800 rpm, is optimal for this incubation. Use of a slower-speed shaker may result in less dense cultures and lower DNA purification yields. Culture-growth should be evaluated by taking OD600 values on a spectrophotometer of 1:10 diluted culture periodically. Cultures should be diluted 1:10 in TB media for this reading. Satisfactory OD600 values for 1:10 diluted culture should range between 0.16-0.25 with the control blank undiluted TB reading between 0.055-0.065.

9) **Pellet cultures.** Spin blocks for 20 min at 4000 rpm (RCF 3315) on the Beckman Avanti J-E centrifuge. After spinning, decant the media from each block into a large bucket or beaker. Blot the decanted blocks, upside-down, on paper-towels spread on the bench-top to remove excess media. Seal each block using an aluminum plate seal and store the blocks at –20°C until needed for DNA purification. The decanted media should be bleached at a final concentration of 5% bleach for 15-30 mins. in the fume hood before being discarded.

10) Solutions 1, 2, and 3 are already made up. They are:

Soln 1: TE Resuspension Buffer
50 mM Tris pH 8.0

10 mM EDTA (8.0)
0.1 mg/mL RNAse
Store at 4°C

Soln 2: NaOH/SDS Lysis Buffer
0.2 M NaOH
1% SDS

Soln 3: KOAC Neutralization Buffer
2.8 M KOAc
Glacial Acetic Acid: added until pH is 5.1
Store at 4°C

11) **Add 200 uL of soln 1** and resuspend: shake at 2000 rpm for 1 minute at room temperature on the Thermomixer. Complete re-suspension of the pellet is necessary for successful lysis. Inadequate re-suspension may result in lower DNA yields. Vortex the block if necessary.

12) **Add 200 uL of soln 2**, seal the plate with an aluminum seal and mix the plate by inverting 4 or 5 times. Carefully time this step from the beginning of soln 2 addition. The lysis time should be 5 mins. Do not exceed 5 mins.

13) **Add 200uL of soln 3**, seal the plate with an aluminum seal and mix the plate by inverting 4 or 5 times. The seal will be loose due to the lysis/neutralization buffers so use caution when inverting.

14) **Spin the block for 20 minutes at 4000 rpm (RCF 3724)** on the Allegra X-15R centrifuge to pellet the lysate.

For Nucleobond anion exchange DNA preparation

15) Solutions N2, N3, and N5 are already made up. They are:

Soln N2: Equilibration Buffer
100 mM Tris
15% EtOH
900 mM KCl
0.15% Triton X-100
Phosphoric Acid: added until pH is 6.3

Soln N3: Wash Buffer
100 mM Tris
15% EtOH
1.15 M KCl
Phosphoric Acid: added until pH is 6.3

Soln N5: Elution Buffer

100 mM Tris
15% EtOH
1 M KCl
Phosphoric Acid: added until pH is 8.5

Note: Successful control of the DNA binding, washing and elution during anion exchange is highly dependent on buffer KCl concentration and pH values. Careful buffer component measurements and pH adjustment is essential. Small deviations from the ascribed measurements can result in a significant loss of yields. Also, all anion exchange buffers should be stored tightly capped to prevent corruption.

16) **Prepare anion exchange resin solution**, before or during the lysate pelleting centrifugation step. Using a 1L bottle fill it with 300ml of Nucleobond resin, and add N2 solution buffer up to 900ml. This step should be done in the hood to protect against silica inhalation.

17) **Place the resin Whatman plate on top of a deep-well block** to act as a waste collection vessel. Mix the anion exchange slurry until it is homogeneous, and then pour out in to a glass trough. Using wide bored P1000 tips and a P1000 multichannel pipette, transfer 450 ul of the slurry into each well 25 um Whatman MBPP filter plate. When done, centrifuge at 750 rpm (RCF 131) for 5 minutes, using any table-top centrifuge Allegra X-15R.

18) **Transfer lysate supernatant to the resin plate** (which is stacked onto an empty waste deep-well block). This is done using the Biomek FX.

19) **Spin the stacked plates for 5 mins. at 300 rpm (RCF 31)** using the Allegra X-15R centrifuge with slow ramp up speed.

20) **Wash column step**. Place stack plate onto the WellMate and add **400- 450 uL of wash buffer N3** to each well. Alternatively, a 1 mL multi-channel pipette may be used to add the wash buffer. Transfer the resin plate to vacuum manifold to remove wash buffer**. Repeat wash steps 4x**. On the last wash make sure all wells are properly emptied. **Spin the stack plate at 750 rpm (RCF 131) for 5 mins.** using Allegra X-15R centrifuge to remove residual wash buffer.

21) **Elution**. Place resin plate onto a clean 800 uL collection plate. Place the stacked plates onto the WellMate and add **300 uL of elution buffer N5** to each well. Let **sit at RT for 10 minutes** then **spin the stacked plates for 5 mins. at 300 rpm** (RCF 131) using the Allegra X-15R centrifuge with slow ramp up speed.

22) Then **spin the stacked plates for 1 min at 1000 rpm (233 RCF). Store plate@-20 C.**

Protocol: Array printing

Material/Equipment	**Amount/well**
Plasmid DNA (from NAPPA DNA prep protocol)	
Sodium acetate (3M, pH 5.5)	
Isopropanol	
80% Ethanol	
384 well plate for arraying, Genetix x7020	
Polyclonal anti-GST antibody (GE Healthcare/Amersham 27457701)	
Polyclonal anti-FLAG antibody (Sigma F7425)	
BS^3 Linker (Pierce 21580)	
Purified GST protein (Sigma G5663)/Flag protein ()	
Whole mouse IgG antibody (Pierce 31204)	
Centrifuge, Eppendorf 5810	
Genetix QArray2 Array Printer	
Silica packets (VWR 100489-246)	
Genetix Bioassay dish dividers (x6026 divider only; x6027 with dish)	
Corning deep bioassay dish (431111)	
Thermo-Fisher WellMate Bulk Liquid Dispenser	
Eppendorf Thermomixer	
Beckman Allegra X15R centrifuge	
Beckman Biomek FX 96/SPAN8 Liquid Handler	

1) **Add 240 uL of isopropanol to each well** of the Plasmid DNA (from the NAPPA prep protocol)

2) **Add 40uL of NaOAc 3M to each well**. Cover the plate with an aluminum seal and mix by inverting 3 times.

3) **Freeze the plates for 15 min at -80^o C.**

4) **Spin at 4000rpm (RCF 3724) for 30 minutes** using the Allegra X-15R centrifuge (***make sure that centrifuge is run at 20^{o}C****, otherwise KCl will precipitate)*. Discard the supernatant.

5) **Add 400-500 uL of 80% ethanol to each well** using WellMate. Seal with aluminum seal and shake at 200rpm for >30mins.

6) Centrifuge at 4000rpm **(RCF 3724) on Allegra X15R centrifuge** for 30 mins. at 20^{o}C. Discard the supernatant.

7) Dry the plate, uncovered for the time the plate needed to be dry that. You should not see any alcohol at bottom of well. Seal and centrifuge at 1000 rpm (233 RCF) on Allegra X15 centrifuge for 2 minutes to bring any pellets down

Array sample preparation:

8) **Prepare master mix**. For one 96-well plate prepare approximately 3 mL of master mix. Master mix contains polyclonal GST antibody (final: 1:100 dilution or 50 ug/mL), Streptavidin (final: 3.6 mg/mL) and BS^3 linker (final: 1.25 mg/mL or 2 mM). You should add the chemicals following the number order.

e.g. Master Mix:

	3ml	10ml
2.*BSA (66 mg/mL stock):	166.5 uL	555ul
4. BS^3 Linker (50 mg/mL stock):	75.0 uL	250ul
3. ** Polyclonal α-GST (5 mg/mL stock):	30.0 uL	100ul
1. AC MQ H_20:	2728.5 uL	9095ul

* Make 10-20 ml of BSA, aliquot to 1mls and store in freezer.
** For FLAG, use 45 ul antibody for every 3 ml of master mix.

9) Using p200 multichannel pipette, transfer 200ul to each well of a PCR plate.

10) Using Biomek FX (NAPPA\Array Preparation*file*), transfer 24ul from the PCR plate to each well of the dry DNA pellet. plates. Spin down and shake at 1000 rpm for 30-40 minutes (DO NOT EXCEED THIS TIME OTHERWISE YOU WILL GET HIGH BACKGROUND).

11) **Transfer all 23 uL to 384 array plate**. This transfer can be done using the Biomek FX or a multi-channel pipette.

12) Spin the plate down briefly (1500 rpm (524 RCF) on Allegra X15R centrifuge for 1 minute – to get rid of bubbles).

13) **Array** *(see below)* using the appropriate array setup and humidity control approximately 60%.

Protocol: Expression of the NAPPA slides

Material/Equipment	**Amount (for3 slides)**
HybriWell gaskets (Grace HBW2160-1LA)	3
Cell free expression system i.e. rabbit reticulocyte lysate (Promega L4610)	1 tube
RNaseOUT (Invitrogen 10777-019)	8 uL
DEPC water (Ambion 9906)	160 uL
SuperBlock (Pierce 37535)	~30 mL
Blocking solution: 5% Milk in PBS with 0.2% Tween20	~120 mL
PBS	
Programmable chilling incubator, with leveling shelves	
Rocking shaker	
Genetix Bioassay dish dividers (x6026 divider only; x6027 with dish)	
Corning deep bioassay dish (431111)	

1) **Block slides: ~1 hr on** rocking shaker at room temperature or 4°C overnight in the coldroom with SuperBlock. Use ~30 mL in a pipette box for 4 slides.

2) **Rinse slides with Milli-Q water**. Dry with filtered compressed air.

3) **Apply HybriWell gasket** to each slide. Use the wooden stick to rub the areas where the adhesive is to make sure it is well stuck to the slide all around.

4) Pre-heat the incubator to be used for IVT at 30^{o}C

5) **Prepare IVT**. Each slide will require **150 uL of IVT lysate mix**. Each tube after component addition will contain 250 uL of lysate mix. Since the lysate tubes cannot be re-frozen, always try to express slides in batches of some multiple of three. Follow the numerical order when adding.

   e.g. 1 tube = 1 slides = 250 uL
   - 3.-5 uL of –Met
   - 2.-45 uL of DEPC water
   - 1.-200 uL of reticulocyte lysate

6) **Add IVT mix from the non-label or non-specimen end to slides**. Pipette the mix in slowly; it's okay if it beads up temporarily at the inlet end. Gently massage the HybriWell to get the IVT mix to spread out and cover all of the area of the array. Apply the small round port seals to both ports.

7) **Place the slides on a bioassay dish** with divider on top of the leveling shelf inside the incubator. Incubate for 1.5 hr at 30^{o}C for protein expression (30 is key; 28 or 32 give reduced yield), followed by 30 min at 15^{o}C for the query protein to bind to the immobilized protein.

8) **Remove the HybriWell and rinse slides twice with PBS 1x** with 0.2% Tween20**.**

9) **Immerse each slide in milk immediately**; wash with milk 3 times, 5 minutes each, in a pipette box. Use about 30 mL milk per wash.

10) **Block slides with milk** on rocking shaker at room temperature for 1 hr.

Protocol: Detection of the NAPPA slides

Material/Equipment	**Amount (for 1 slide)**
Primary AB, mouse anti-GST (Cell Signal 2624)	150 uL of stock solution
Primary AB, mouse anti-HA	150 uL of stock solution
Secondary AB, HRP-conjugated anti-mouse (Amersham NA931)	150 uL of stock solution
Secondary AB, HRP-conjugated anti-mouse (Jackson Lab Cat #515-035-062)	
Secondary AB, HRP-conjugated anti-human (Jackson Lab Cat #109-035-098)	
TSA reagent (PerkinElmer SAT704B001EA)	150 uL of stock solution
Milk (5% Milk in PBS with 0.2% Tween20)	90 mL for 4 slides at once
PBS (pH 7.4)	90 mL for 4 slides at once
Coverslips, 24 x 60 mm (VWR 48393-106)	3
Lifterslips, 24 x 65 mm (Erie 25X65I-2-5251-001-LS)	3
Pipette boxes	1
Scanner, Tecan PowerScanner	

1) If needed, prepare antibody solutions in PBS milk 5%: 1:200 mGST, 1:1000 HA, 1:200 anti-mouse, 1:500 anti-human IgG. Store at 4°C.

2) **Apply primary AB** (mouse anti-GST, mouse anti-HA or mouse anti-Flag (1/200)) by adding 600 uL to the non-label or non-specimen end of the slide. Treat each slide one at a time and ensure that the slides do not dry during this step. **Incubate for 1 hr at RT; wash with milk on a rocking shaker** (3 times, ~ 5 min each). Drain.

   For serum or antibody screening, dilute your serum at 1:200 in 2ml 5%milk/PBST. Place in corning chamber and incubate at 4^{o}C overnight with end-over-end rotation. When done the next morning, wash the slides 3x 5 minutes with milk.

3) **Apply secondary AB**: For serum screening, you will need 2ml of 1:500 HRP-conjugated anti-human antibody (from Jackson Labs) for each slide. Place in corning chamber and add 2ml of you secondary antibody, **Incubate for 1 hr at RT**; **wash with PBS 1x** with 0.2% Tween20 **3 times, ~ 5 min each. Quickly rinse with Milli-Q water. Drain**.

4) **Before applying TSA solution, make sure slides are not too wet**, but don't let them fully dry. (If they are too wet, it will dilute the TSA). **Apply 500 uL TSA** mix and place coverslip**. Incubate for 10 minutes at room temperature**. Rinse in Milli-Q water; dry with filtered compressed air.

5) Scan.

Protocol: Detection of the DNA on NAPPA slides

Material/Equipment	**Amount (for 4 slides)**
PicoGreen (Invitrogen P11495)	
PicoGreen stock solution	33 uL
SuperBlock	50 mL
PBS (pH 7.4)	150 mL
Coverslips, 24 x 60 mm	4
Lifterslips, 24 x 65 mm	4
Rocking shaker	
Scanner, Tecan PowerScanner	

1) Block the slides with SuperBlock on a rocking shaker for 1 hour.
2) If necessary, prepare PicoGreen stock solution: to the 100 uL/vial that comes, add 200 uL TE buffer, then do a 1:600 dilution in SuperBlock (i.e. for 4 slides, add 33 uL PicoGreen stock solution to 20 mL SuperBlock).
3) For a single slide, small array: apply 150 uL PicoGreen mix, and apply coverslip. Let sit for 5 minutes at room temperature. For 4 slides, add 20 mL in a box and shake on rocking shaker for 5 minutes.
4) Wash with PBS (pH 7.4) 3 times, ~ 5 min each. Quickly rinse with Milli-Q water.
5) Dry with filtered compressed air.
6) Scan.