Agilent Protein Binding Microarrays (4 x 44k):

Updated 2/12/14 - LA

***** STERILE FILTER ALL BUFFERS TO AVOID DUST ON ARRAYS*****

	Final Concentration	10x PBS	Milli-Q H_20	Other
1x PBS	---	100ml	900ml	---
Solution A	0.01% TX-100 / PBS	100ml	899ml	1ml 10% TX-100 (in H_2O)
Solution B	0.1% Tween-20 / PBS	100ml	895ml	5ml 20% Tween-20 (in H_2O)
Solution C	0.5% Tween-20 / PBS	100ml	875ml	25ml 20% Tween-20 (in H_2O)
Solution D	0.05% Tween-20 / PBS	100ml	897.5ml	2.5ml 20% Tween-20 (in H_2O)

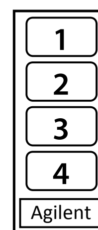
1. Dissolve milk solutions on shaker for 2 hours (200rpm), syringe filter 0.45um and keep on ice
    a. 2% milk: 0.1g powdered milk in 5ml 1X PBS
    b. 4% milk: 0.1g powdered milk in 2.5ml 1X PBS
2. Prepare Protein Binding Mixture:
    a. Thaw protein and reagents on ice, label tube with protein and array number

    PROTEIN-BINDING MIXTURE:

18.6ul	Milli-Q H_2O (sterile)
15.7ul	4X PBS (sterile)
62.5ul	4% milk in 1X PBS (sterile)
0.7ul	Salmon testes DNA (~53 ng/ul stock)
2.5ul	10 mg/ml BSA (0.2 ug/ul final conc.)
25ul	Protein (in elution buffer with 25% glycerol)
125ul	

    b. Flick tube to mix and spin briefly to get rid of bubbles
    c. Incubate mixture on ice for 1 hour
        i. *Note - leftover protein cannot be re-frozen or used again*
        ii. *If working with multiple samples, make a master-mix of all reagents except protein*
3. Block for non-specific binding:
    a. Pre-wet double-stranded array in **Solution A** in Coplin jar (max. 2 arrays per jar)
        i. Shake on platform at 125rpm for 5 min (cover with foil)
    b. Add 800ul 2% milk to single-chamber gasket slide positioned in steel hybe chamber
        i. Use pipette tip to spread the milk evenly over the well without touching gasket
    c. Fill a staining dish with 1X PBS (use throughout the entire protocol, keep covered)
    d. Rinse array briefly in PBS and pull out of solution slowly to remove droplets
    e. Lower array ("Agilent" side facing down) onto the gasket and close hybe chamber
        i. Be careful to avoid bubbles and avoid wetting the top of the gasket
    f. Block array in 2% milk for 1 hour in the dark (cover the chamber with foil or ice bucket)
4. Wash off blocking agent:
    a. Fill a staining dish and Coplin jar with **Solution B**
    b. Remove gasket/slide sandwich, taking care not to break the seal
    c. Disassemble while submerged in Solution B using plastic tweezers
    d. Shake slide underwater and quickly transfer to Coplin jar (shake @ 125rpm for 5 min)
        i. *Rinse staining dish and gasket slide with water, spin slide dry and return to box*
    e. Repeat Coplin jar wash step using **Solution A** (shake 2 min)

5. Bind Protein:
    a. Prepare steel hybe chamber with 4-well gasket slide, note orientation:
    b. Pipette 125ul protein binding mixture into each well
        i. *Note - it is best to use 2 non-adjacent wells per slide (i.e. 1 + 3 OR 2 + 4) to minimizes the damage done if one of the gaskets leaks*
    c. Remove slide from Coplin jar, rinse briefly in PBS as before and remove slowly
    d. Lower array ("Agilent" side facing down) onto the gasket slide and close hybe chamber
    e. <u>Incubate protein binding mixture on array for EXACTLY 1 hour in the dark</u>



6. During the protein binding step, prepare fluorescent antibody mixture (will need 125ul/array):

FLUORESCENT ANTIBODY MIXTURE:

# of Arrays	2% Milk	Alexa488-conjugated anti-GST antibody (4°C)	Total Vol.
1	125.8ul	3.2ul	129ul
2	248.6ul	6.4ul	255ul
3	370.5ul	9.5ul	380ul
4	492.4ul	12.6ul	505ul

    a. <u>Wrap tube in foil and incubate antibody mixture on ice for 1 hour</u>
    b. *This is a 1:40 dilution of primary antibody (Molecular Probes #A-11131, 2 mg/ml stock).*
7. Wash off protein binding mixture:
    a. Fill a staining dish and Coplin jar with **Solution C**
    b. Remove gasket/slide sandwich, taking care not to break the seal
    c. Disassemble while submerged in Solution C using plastic tweezers
    d. Shake slide underwater and quickly transfer to Coplin jar (shake @ 125rpm for 3 min)
    e. Repeat Coplin jar wash step using **Solution A** (shake 2 min)
        i. *Wash 4-well gasket slide with water and spin slide dry*
8. Bind Fluorescent Antibody:
    a. Prepare hybe chamber with dried 4-well gasket slide and 125ul antibody mixture
    b. Remove slide from Coplin jar, rinse briefly in PBS as before, removing slide from PBS slowly
    c. Lower array ("Agilent" side facing down) onto the gasket and close hybe chamber
    a. <u>Incubate fluorescent antibody mixture on array for 1 hour in the dark</u>
9. Wash off fluorescent antibody mixture:
    a. Fill a staining dish and <u>two</u> Coplin jars with **Solution D**
    b. Remove gasket/slide sandwich, taking care not to break the seal
    c. Disassemble while submerged in Solution D using plastic tweezers
    d. Shake slide underwater and quickly transfer to Coplin jar (shake @ 125rpm for 3 min)
    e. Repeat Coplin jar wash step using fresh **Solution D** (shake 3 min)
    f. Repeat Coplin jar wash step using **1x PBS** (shake 2 min)
    g. Rinse briefly in PBS staining dish as before, pulling up slowly to remove liquid droplets
        i. Spin array dry if droplets remain
10. Store array slide in a slide box in the dark until ready to scan

Note: PBM replicates should be run on different versions – same probes, different arrangement

Scanning PBM Arrays

1. Scan arrays using an Axon 4300A scanner or similar (requires 488nm blue laser)
   a. Wavelength #1: 488nm
   b. Pixel Size: 5um
   c. PMT Gain: 350
   d. Power (%): 100
   e. Filter: Standard Blue
   f. Lines to Average: 1
2. Scan full slide at low resolution to preview using "**>>**" button
3. Select single array and scan at high resolution using "**>**" button
   a. Use "**!**" button to auto-contrast image
   b. Zoom in to check Simple Line Profile
      i. Click on Measuring Tools (pencil icon) and select Simple Line Profile
      ii. Draw a line across a row of spots with at least one bright (saturated) spot
      iii. Graph should show a large peak representing bright spot
   c. Once you get saturated white spots (around 60,000), Save Image – Selected Scan Area
      i. [20130325_v11#417_ProteinName_Alexa488_PMT350.tif]
   d. Repeat scan at lower PMT Gain using the Simple Line Profile readout as a guide
      i. Highest scan peak = 60,000
      ii. Middle scan peak = 40,000
      iii. Lowest scan peak = 5,000-10,000
4. Gene Pix Pro software:
   a. Open lowest PMT scan and import settings file from double-stranded scan (.gps file)
   b. Align the grid and flag spots for removal
   c. Save Settings As [20130325_v11#417_ProteinName_Alexa488.gps]
   d. Click "Analyze" to generate a results file for each of the 4 arrays
   e. Repeat above steps for the middle and high-PMT scans

For more information: **Berger MF and Bulyk ML. Universal protein-binding microarrays for the comprehensive characterization of the DNA-binding specificities of transcription factors. *Nature Protocols.* (2009) 4(3):393-411**

Folder: PBM FILES > v11#417

File	Description
20130125_v11#417_Cy3.gps	*Double stranding settings file (grid alignment and flagged spots)*
20130125_v11#417_Cy3_PMT350.tif	*Double stranding high-res scan at 350 PMT*
20130125_v11#417_Cy3_PMT350_1-4.gpr	
20130125_v11#417_Cy3_PMT350_2-4.gpr	*Double-stranding fluorescent values for each spot including info on what was flagged*
20130125_v11#417_Cy3_PMT350_3-4.gpr	
20130125_v11#417_Cy3_PMT350_4-4.gpr	

Folder: PBM FILES > v11#417 > v11#417-1 ProteinA

File	Description
20130125_v11#417_ProteinA_Alexa488.gps	*Alexa488 settings file (grid alignment and flagged spots)*
20130325_v11#417_ProteinA_Alexa488_PMT350.tif	*Alexa488 high-res scan at 350 PMT*
20130325_v11#417_ProteinA_Alexa488_PMT300.tif	*Alexa488 high-res scan at 300 PMT*
20130325_v11#417_ProteinA_Alexa488_PMT250.tif	*Alexa488 high-res scan at 250 PMT*
20130325_v11#417_ProteinA_Alexa488_PMT350_1-4.gpr	*Alexa488 quantitative data for array 1*
20130325_v11#417_ProteinA_Alexa488_PMT350_2-4.gpr	*Alexa488 quantitative data for array 2 (junk)*
20130325_v11#417_ProteinA_Alexa488_PMT350_3-4.gpr	*Alexa488 quantitative data for array 3 (junk)*
20130325_v11#417_ProteinA_Alexa488_PMT350_4-4.gpr	*Alexa488 quantitative data for array 4 (junk)*
20130325_v11#417_ProteinA_Alexa488_PMT300_1-4.gpr	*Alexa488 quantitative data for array 1*
20130325_v11#417_ProteinA_Alexa488_PMT300_2-4.gpr	*Alexa488 quantitative data for array 2 (junk)*
20130325_v11#417_ProteinA_Alexa488_PMT300_3-4.gpr	*Alexa488 quantitative data for array 3 (junk)*
20130325_v11#417_ProteinA_Alexa488_PMT300_4-4.gpr	*Alexa488 quantitative data for array 4 (junk)*
20130325_v11#417_ProteinA_Alexa488_PMT250_1-4.gpr	*Alexa488 quantitative data for array 1*
20130325_v11#417_ProteinA_Alexa488_PMT250_2-4.gpr	*Alexa488 quantitative data for array 2 (junk)*
20130325_v11#417_ProteinA_Alexa488_PMT250_3-4.gpr	*Alexa488 quantitative data for array 3 (junk)*
20130325_v11#417_ProteinA_Alexa488_PMT250_4-4.gpr	*Alexa488 quantitative data for array 4 (junk)*