Protocols:

Trainee: Anna Vickrey
Host lab: Shapiro Lab, University of Utah
Dates of Internship: 06/26/2012- 09/1/2012

Title: Gibson Cloning (from Dr. Eric Domyan)
Rationale and background: To ligate insert and vector fragments for high efficiency plasmid transformations.
Protocol:
I: Quick Protocol
1. Thaw mix on ice.
2. Split into two tubes (7.5ul each)
3. Add 2.5ul total DNA (target 1:1:1... molar ratios but this is not critical)
4. Incubate at 50C for 1hr
5. Transform 2 ul in 50-100 ul of cells.
*If you want to chew up methylated background DNA add .5 ul DpnI and Incubate at 37 for 15 -30 min
II: Gibson reaction from set up:
1. Prepare 5X ISO buffer. Six ml of this buffer can be prepared by combining the following:
    3 ml of 1 M Tris-HCl pH 7.5
    150 µl of 2 M MgCl2
    60 µl of 100 mM dGTP
    60 µl of 100 mM dATP
    60 µl of 100 mM dTTP
    60 µl of 100 mM dCTP
    300 µl of 1 M DTT
    1.5 g PEG-8000
    300 µl of 100 mM NAD
    Add water to 6 ml
2. Aliquot 100 µl and store at -20 °C
3. Prepare an assembly master mixture. This can be prepared by combining the following:
    320 µl 5X ISO buffer
    0.64 µl of 10 U/ µl T5 exonuclease
    20 µl of 2 U/µl Phusion polymerase
    160 µl of 40 U/µl Taq ligase
    Add water to 1.2 ml
4. Aliquot 15 µl and store at -20 °C. This assembly mixture can be stored at -20 °C for at least one year. The enzymes remain active following at least 10 freeze-thaw cycles.
This is ideal for the assembly of DNA molecules with 20-150 bp overlaps. For DNA molecules overlapping by larger than 150 bp, prepare the assembly mixture by using 3.2 µl of 10 U/ µl T5 exo.
5. Thaw a 15 µl assembly mixture aliquot and keep on ice until ready to be used.
6. Add 5 µl of DNA to be assembled to the master mixture. The DNA should be in equimolar amounts. Use 10-100 ng of each ~6 kb DNA fragment. For larger DNA segments, increasingly proportionate amounts of DNA should be added (e.g. 250 ng of each 150 kb DNA segment).
7. Incubate at 50 °C for 15 to 60 min (60 min is optimal).
8. If cloning is desired, transform 2 µl of the reaction mixture into 50-100 µl competent cells (DH5α) by heat shock

Title: RNA in situ hybridization (from Dr. Eric Domyan)
Rationale and background: To visualize gene expression by binding a tagged probe to target mRNA
Protocol:

DAY 0 – Dissect tissue, fix in 4% paraformaldehyde (4% PFA) at 4°C overnight. Dehydrate through methanol gradient into 100% MeOH at 4 °C, store at -20°C.

DAY 1 – All incubation steps to be performed with gentile agitation on an orbital shaker, unless otherwise indicated.

1. Prepare supplies and preheat solutions.
- **A.** Transfer prehyb stock solution into sealed conical tube and preheat to 60.5°C.

2. Rehydrade fixed tissue (In 6-well baskets or nunc tubes. Use 5mL/wash for baskets and 2mL/wash for nunc tubes.)
- **A.** Wash tissue 1 x 15 min each in 75%MeOH/25% PBSTw, 50%MeOH/50%PBSTw, 25% MeOH/75% PBSTw.
- **B.** Wash tissue 2 x 15 min in PBSTw.

3. Incubate tissues for 30 min at 25°C in 2mL/well of 6% H_2O_2.
4. Wash tissues 4 x 5 min at 25°C with 2mL/well PBSTw.
5. Incubate tissues 12 min at 25°C in 2mL/well proteinase K solution.
6. Wash tissues 1 x 5 min at 25°C with 2mL/well PBSTw.
7. Incubate tissues 20 min at 25°C in 2mL/well post-fix solution.
8. Wash tissues 2 x 5 min at 25°C with 2mL/well PBSTw.
9. Transfer tissues into separate 2mL Nunc tubes, 1 tube per probe.
10. Prehybridization step: Incubate for at least 1 hr at 60.5°C in 1.5 to 2mL prehyb buffer.
11. Hybridization step: Add 0.65mg probe/well and incubate sections overnight in hybridization chamber at 60.5°C in probe+prehyb buffer.
12. Prepare and preheat solutions for Day 2: Add SDS to solution 1 (to a final concentration of 1%) and preheat overnight at 60.5°C.

DAY 2 - All incubation steps to be performed with gentile agitation on an orbital shaker, unless otherwise indicated.

1. Transfer tissue into baskets of a 6 well plate. Remove hyb buffer from each well and wash tissues 3 X 30 min at 60.5°C with 5mL/well Solution 1.
2. Prepare and preheat solutions.
- **A.** Prepare 50/50% mix of Solution1/Solution 2 and preheat to 60.5°C.
- **B.** Add RNase to Solution 2 and preheat to 37°C.
- **C.** Preheat one wash volume of Solution 3 to 25°C and two wash volumes to 60.5°C.

3. Wash tissues 1 X 10 min at 60.5°C with 5mL/well of 50/50% mixture of Solution 1/ Solution 2.
4. Wash tissues 4 X 10 min at 25°C with 5mL/well Solution 2.
5. Incubate tissues 15 min at 37°C in 5mL/well RNase solution.
6. Wash tissues 1 X 10 min at 25°C with 5mL/well Solution 2.
7. Wash tissues 1 X 10 min at 25°C with 5mL/well Solution 3.
8. Wash tissues 2 X 1 hr at 60.5°C with 5mL/well Solution 3.
9. Preheat Tissue Blocking (TB) buffer to 25°C.
10. Wash tissues 3 X 10 min at 25°C with 2ml/well TBSTw.
11. Transfer tissues into new 2mL Nunc tubes.
11. Blocking step
- **A.** Incubate tissues at least 2 hr at 25°C in 2mL/well TB buffer.

12. Antibody step
- **A.** Dilute anti-DIG AP antibody 1:2000 in TB buffer.

- **B.** Add 2 mL of TB containing the antibody to each Nunc tube.
- **C.** Incubate tissues overnight at 4°C in 2mL TB buffer + antibody.

Day 3 - All incubation steps to be performed with gentile agitation on an orbital shaker, unless otherwise indicated.

1. Remove antibody solution and save (can be reused).
- **A.** Add sodium azide to a final concentration of 0.2mM to prevent microbial growth.
- **B.** Store antibody solution at 4°C and reuse up to two additional times.

2. Transfer tissues into baskets of 6 well plate.

3. Wash tissues 8 X 30 min at 25°C with 5mL/well TBSTw containing 2 mM levamisole.

4. Remove tissues from baskets, use forceps to separate sections & remove visible debris, transfer tissues into clean Nunc tubes.

5. Wash tissues 1 X 10 min at 25°C with 1.5mL NTMT containing 2 mM levamisole.

6. Detection step
- **A.** Protect tissues from light and incubate at 25°C in 1mL/tube of a 60/40% mixture of NTMT containing 2mM levamisole/BM Purple.
- **B.** Color development time ranges from several hours to several days. Change NTMT/BM Purple solution as needed (substrate will precipitate over time).
- Once color is fully developed, wash tissues 2 X 5 min at 25°C with 1mL/tube NTMT containing 2mM levamisole.

7. Bleaching step
- **A.** Post-fix tissues overnight at 4°C in 1mL/tube 4% PFA.
- **B.** Remove PFA and incubate tissues for 30 min at 25°C in 1mL/tube PBSTw containing 3% H2O2.
- **C.** Wash tissues 1 X 10 min at 25°C in 1mL/tube PBSTw.
- **D.** Store tissues at 4°C in 1mL/tube 4% PFA.

Solutions

Day 1

PBSTw: 1X PBS containing 0.1% Tween-20 and 0.2mM sodium azide. Pass through 0.2µm filter to remove insoluble material/contaminants.

6% H_2O_2: 1mL 30% H_2O_2 per 4mL PBS

Proteinase K: 0.25µL 20mg/mL proteinase K per 1mL PBSTw

Post-fix: 8µL 25% glutaraldehyde per 1mL 4% PFA

Prehybridization solution

stock solution	**final conc**	**for 1000mL**
100% formamide	50%	500mL
20X SSC	5X	250mL
Blocking reagent	1%	10g
10mg/mL yeast tRNA	10µg/mL	1mL
10mg/mL heparin	10µg/mL	1mL
dH_2O to vol.	--	to 1000mL

Aliquot 50mL volumes into conical tubes & store at –20°C.

Solution 1

stock solution	**final conc**	**for 500mL**

100% formamide	50%	250mL
20X SSC	5X	125mL
dH_2O	--	75mL
10% SDS	1%	50mL

Mix formamide, SSC & dH_2O as shown above & store at –20°C.
DO NOT add SDS to freezer stock solution because SDS will precipitate in the cold.
Prior to using Solution 1 for ISH, add 1mL of 10% SDS per 9mL Solution 1 stock.

Day 2

Solution 2

stock solution	**final conc**	**for 500mL**
1M Tris-HCl, pH 7.5	10mM	5mL
5M NaCl	0.5M	50mL
100% Tween-20	0.1%	0.5mL
0.2M sodium azide	0.2mM	0.5mL
dH_2O	--	444mL

Pass through 0.2µm filter to remove insolubles/contaminants, store at 25°C.
RNase: 5µL RNase (50µg/mL) per 1mL Solution 2

Solution 3

stock solution	**final conc**	**for 500mL**
20X SSC	2X	50mL
100% formamide	50%	250mL
dH_2O	--	200mL

Store at –20°C

Tissue blocking (TB) buffer

stock solution	**final conc**	**for 500mL**
10X TBS	1X	50mL
100% sheep serum	10%	50mL
10% blocking reagent	1%	50mL
BSA	1%	0.5g
dH_2O to vol.	--	to 500mL
100% Tween-20	0.1%	0.5mL

Mix TBS, serum, blocking reagent, BSA & dH_2O as shown above.
Filter through #2 Whatman filters.
Add Tween-20.
Aliquot 6mL volumes into conical tubes & store at –20°C.

Sheep serum (must be heat-inactivated before use).
To heat inactivate:

- thaw new bottle of serum
- incubate at 70°C for 30 min
- aliquot & store at –20°C

10% Blocking reagent

stock solution	final conc	for 100mL
maleic acid	100mM	1.2g
5M NaCl	150mM	3mL
dH_2O to vol.	--	to 100mL
Blocking reagent	10%	10g

Mix maleic acid, NaCl & dH_2O according to above, pH to 7.5 (note: strong buffer so difficult to pH, try using solid NaOH pellets to raise pH initially).
Add blocking reagent, microwave briefly to aid solubility (avoid boiling over, solution will be cloudy & viscous so watch carefully to ensure blocking reagent is completely dissolved in solution).
Aliquot 10 mL volumes into conical tubes & store at –20°C.

Day 3

2M Levamisole: Dissolve 4.82g levamisole in ~7mL double-distilled H_2O (total volume should equal 10mL), aliquot 200µL volumes & store stocks at –20°C.
TBSTw + levamisole: 1X TBSTw containing 2mM levamisole.
NTMT + levamisole (inhibits endogenous alkaline phosphatases):

stock solution	**final conc**	**for 500mL**
1M Tris-HCl, pH 9.5	100mM	50 mL
5M NaCl	100mM	10mL
1M $MgCl_2$	50mM	25mL
dH_2O	--	415mL
100% Tween-20	0.1%	0.5 mL
2M Levamisole	2mM	0.5mL

Mix Tris, NaCl, $MgCl_2$ & dH_2O as shown above.
Pass through 0.2µm filter to remove insoluble material/contaminants. Store at 25°C.
DO NOT add Tween or levamisole to stock solution.
Prior to using NTMT for ISH, add 1µL of 100% Tween-20 and 1µL of 2M levamisole per 1mL NTMT stock.
3% H_2O_2: 1mL 30% H_2O_2 per 9mL PBSTw.

Abbreviations

BSA = bovine serum albumin
DIG = digoxigenin
H_2O_2 = hydrogen peroxide
PFA = paraformaldehyde
PBS = phosphate-buffered saline
PBSTw = 1X PBS + 0.1% Tween-20

SDS = sodium dodecyl sulfate (aka lauryl sulfate)
SSC = saline sodium citrate
TBS = Tris-buffered saline
TBSTw = 1X TBS + 0.1% Tween-20

Reagents and Supplies

Anti-DIG antibody, Fab fragments, cat # 11214667001, Roche
Blocking reagent, cat # 11096176001, Roche
BM Purple AP substrate, precipitating, cat # 11442074001, Roche
BSA, cat # BP1600-100, Fisher
Formamide, cat # F5786-1L, Sigma
Glutaraldehyde, 25% solution in H_2O, cat # G6257-100ML, Sigma
Heparin, sodium salt, cat # H3393, Sigma
Hydrogen peroxide, 30% solution in H_2O, cat # BP2633-500, Fisher
Levamisole, cat # L9756, Sigma
Magnesium chloride, cat # M33-500, Fisher
Maleic acid, cat # M0375-500G, Sigma
Paraformaldehyde, cat # 101176-014, VWR
PBS, w/out Ca & Mg, MP Biomedicals powdered media, cat # ICN1760420, Fisher
Proteinase K solution, 20mg/mL, biotechnology grade, cat # E195-5ML, Amresco
RNase, cat # R6513, Sigma
SDS, cat # S529-500, Fisher
Sheep serum, cat # S2263-500mL, Sigma
Sodium azide, granular, cat # S227I-100, Fisher
Sodium chloride, cat # BP358-212, Fisher
SSC, 20X solution, cat # S24022-4000.0, Research Products International
Tris-HCl, cat # BP153-1, Fisher
Tween-20, cat # BP337-100, Fisher
Yeast tRNA, cat # 109495, Roche
Polyester mesh, 33 micron, 12" x 24", cat # CMY-0033-D, Small Parts Inc.

Title: Bacterial Toxicity Assay (from Dr. Eric Domyan)
Rationale and background: Test the toxicity (to *E. coli*) of IPTG-induced expression of different plasmid inserts.
Protocol:

1. For each construct you want to test, add 15 mL LB + 15uL 100mg/mL carbenicillin to a 15mL culture tube.
2. Pick a single colony from a plate with a micropipette tip; swirl tip in your tube of LB+ carb.
3. Incubate tube of LB at 37°C with shaking at 300rpm for 1 hr.
4. Split the culture into 6 x 2 mL aliquots in 15 mL culture tubes.
5. To 3 of the tubes, add 45uL 100mg/mL IPTG.
6. Incubate tubes O/N with shaking at 37°C.
7. The next day, determine the OD600 with a spectrophotometer.

   LB recipe: pH=7.0 (1L of ddH2O)
   10g NaCl
   10g Bacto Tryptone
   5g Yeast Extract

   1. Fill container with 750L of ddH2O

2. Weigh out ingredients
3. Use stir bar to dissolve everything into H2O.
4. pH to 7.0 with 10M NaOH (approx. 1 drop)
5. Bring volume up to 1L
6. Pour solution into 2-500ml bottles
7. Label solution and date
8. Put autoclave tape on and autoclave for 40 min.

Title: Assay for soluble protein expression (from Dr. Martin Horvath)
Rationale and background: Grow cells containing a construct to test for the expression of soluble GST-tagged protein in *E. coli*. Expect some optimization (induction times and temperatures, buffers, cell growth at induction, etc.). Here, we tested protein expression in 60mL cultures under two induction conditions: 20 hours at 20°C and 3 hours at 37°C. You will need to grow separate 60mL cultures for each of your induction conditions. Use BL21 pLysS strain.
Protocol:

1. Start a 2mL liquid culture from a colony containing the GST-construct that you plan to induce expression in. Grow overnight (O/N) shaking at 180 rpm at 37°C in 2mL LB, 3.7uL 100mg/mL carbenicillin (carb), and 2uL 68mg/mL chloramphenicol (chlor).
2. The next morning, wash the cells and use them to inoculate a 60mL 2xYT culture in a **baffled** flask. Before inoculating, set aside an aliquot (~1mL) of 2xYT to use as a spectrophotometer blank.
    - Spin the 2mL O/N culture at 4750rpm at 4°C for 5 min.
    - Pour off the media, and borrow 2mL 2xYT to re-suspend cells.
    - Spin the 2mL culture at 4750rpm at 4°C for 5 min. Pour off media.
    - Re-suspend again in 2mL borrowed 2xYT, transfer to inoculate ~60mL 2xYT culture with the washed cells.
3. Grow ~60mL culture at 37°C with shaking.
4. Monitor the OD at 600nm starting at least 1 hr. after inoculation.
5. When OD=1 (0.85-1.2 will do), at the same time:
    - Induce expression by adding 300uL IPTG.
    - Move culture to desired expression conditions (e.g. 20hr. at 20°C).
6. After induction, spin down 45mL culture by centrifugation for 10 min at 4750 rpm, 4°C.
7. Pour off the supernatant and re-suspend in 2mL Cell Wash with 7.5uL 0.5M PMSF.
8. Divide into three aliquots:
    - 60uL (After freezing, lyse and run on an SDS-PAGE gel to visualize crude extract)
    - 2X 0.97mL (After freezing, sonicate and purify GST-tagged protein by Batch method, then run on an SDS-PAGE gel to visualize soluble GST-tagged protein expression).
9. Spin down aliquots by centrifugation at room temperature at 6000 rpm.
10. Remove supernatant and freeze at -20°C O/N.

Solutions:

1. LB: see “Toxicity Assay”
2. 2xYT media:
    1. Measure ~900ml of distilled H_2O.
    2. Add 16g Bacto Tryptone.
    3. Add 10g Bacto Yeast Extract.
    4. Add 5g NaCl.
    5. Adjust pH to 7.0 with 5N NaOH.

6. Adjust to 1L with distilled H_2O.

Sterilize by autoclaving.

On the same day that you start a 60mL culture, add 1.8 mL 1 M potassium phosphate pH 7.8, 300uL 1M glucose, 120uL 100mg/mL carb, 60uL 68mg/mL chlor to 60mL 2xYT media.

3. Cell Wash (10 mM Tris pH 8.2, 100 mM NaCl, 1 mM EDTA) makes 500 mL
   Combine at room temperature; bring to 500mL total volume with di-H_2O:
   - Tris base 0.61 g
   - 5 N hydrochloric acid (HCl) 0.5 mL
   - 0.5 M EDTA 1.0 mL

   Do not add PMSF to stock Cell Wash.