Protocols

∅∅∅∅∅ **Autoclaving**

This protocol is based on a protocol by Benjamin Parker.
This document is version 1.0. Last updated: 1/21/2013.

Autoclaving is a process by which high pressure steam is used to clean and sterilize lab equipment and solutions, as well as break down nutrient rich tissues that could become a biohazard. The steam, which is kept at 121°C, is far above the boiling point of water and is consequently fatal to most microbes and viruses. Despite this, the process has no effect on prions or some strains of archaea capable of surviving higher temperatures.

This protocol is written with specificity for the autoclave machine in room 3152. If a different autoclave is being used, please update this protocol.

Note that the methods for glassware sterilization will require some kind of liquid be placed in the bottom of the containers being sterilized-this can keep the container from fracturing in the heat, if composed of glass. In the normal case of sterilizing glassware after washing, this will be distilled water; in the process of making stocks of nutrient broths, this will be LB solution. The solution cannot contain significant amounts of mineralized (“hard”) water, however, as this will cause boiling over.

Materials
Autoclave tape
Autoclave tray
Thick, heat resistant gloves (NOT LATEX GLOVES!)

You will also need access to an
Autoclave

Procedure

1. You can only autoclave liquids or solids together at one time. Do not autoclave liquids and solids at the same time. **Warning**: certain plastics can melt. Orange glassware caps and the special plastic autoclave trays are the exceptions.

Solids (Gravity Cycle)	Liquids (Liquid Cycle)
Pipette tips, empty glassware, etc...	Solutions, Media, Waste, etc...
~30 minutes run time	~50-60 minutes run time

2. There is a foot pedal on the machine that controls the door. Always wear closed toe shoes when autoclaving.
3. If autoclaving solids, place them in the tray, put them in the autoclave, and select a Gravity cycle. Make sure they have autoclave tape on them.

   ⇒Folding the end of a piece of autoclave tape so it sticks to itself makes a little "handle" allowing for easy removal of the tape in the future.
4. If autoclaving liquids, screw the caps on **loosely**. Otherwise your bottles may explode. :)

After the process is complete, remove the tray with heat resistant gloves. Everything will be very, very hot!
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End Autoclaving ∅∅∅∅∅
∅∅∅∅∅ **Bacterial Culture**
This protocol is original by Mike Ferguson
This document is version 1.0. Last updated: 1/21/2013.

Solutions
Sterile LB, SOB, or SOC media
Your favorite antibiotic

Materials
10mL glass tubes

You will also need access to a
Shaking incubator

Procedure
1. Aliquot 5mL of sterile media to an empty, sterile 10mL glass tube.
2. Add the necessary antibiotic. **If you do not add antibiotics, you will end up with a large population of cells that do not have the plasmid.** The antibiotic aliquots should be set up so that you should need to add 5µL of the antibiotic for every 5mL of media.
3. Add your colony to the media; either by dropping a toothpick, that you stabbed a colony with, into the 10mL glass tube or by dropping a pipette tip in that you sucked up some bacterial culture with.
4. Place the tubes into a shaking incubator at 37°C, 150rpm, for approximately 16 hours or until turbid. Growth times may vary depending on plasmid copy number, protein overexpression, etc...

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End Bacterial Culture ∅∅∅∅∅

∅∅∅∅∅ **Chemically Competent Cell Production**
Based off of the TOP10 chemically competent cells protocol.
version 1.0 Updated:1/21/2013

Store competent cells at -80°C only! Leaving the cells at room temperature for any extended period of time will completely destroy competency. Never reuse competent cells. If they have been thawed once before, throw them away.

Solutions
CCMB80 Buffer

Materials
250mL Erlenmeyer flask
25mL Serological pipettes
50mL conical plastic tubes

You will also need access to a
Large Benchtop centrifuge

1. Inoculate 5mL LB with your favorite strain of bacteria for subculturing competent cells. Grow them for ~16 hours at 37°C. The goal is to just get a population of cells in a uniform state.
2. Add 1mL of the bacterial culture to an Erlenmeyer flask containing 100mL sterile SOB.
3. Grow to an optical density of .3 absorbance units at 600nm. This takes ~2 hours. Will vary with strains.
4. Aliquot the cells into 50mL conical plastic tubes. Spin down the cells at 4500rpm for 10 min at 4°C. Decant the supernatant.
5. For every 50mL worth of cells, **gently** resuspend them in 16mL of **ice chilled CCMB80** buffer.

   ⇒You will have to recalculate amounts if you are not working with 50mL aliquots of cells. Just use the

ratios given.

6. Incubate the cells on ice for 20 minutes.
7. Centrifuge the cells again at 4500rpm for 10 min at 4°C. Decant the supernatant.
8. For every 50mL worth of cells, gently resuspend them in 2mL of ice chilled CCMB80 buffer.
9. Immediately aliquot the cells into 1.5mL tubes and store at -80°C. 200µL aliquots usually work well. The CCMB80 will kill the cells if they are left at room temperature for an extended period of time.

Never reuse competent cells. If they have been thawed once before, throw them away.

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End Chemically Competent Cell Production ∅∅∅∅∅

∅∅∅∅∅ **Chemical Transformation**
modified from NEB transformation protocol.
version 1.0 Updated:1/21/2013

Store competent cells at -80°C only!

6. Thaw frozen competent cells on ice.
7. Chill approximately 5ng (2µL) of the ligation mixture or DNA in a 1.5mL microcentrifuge tube. If doing a **co-transformation**, just add 2µL of the other plasmid for a total of 4µL DNA mixture.
8. Add 100µL of competent cells to the DNA. Mix gently by pipetting up and down or flicking the tube 4-5 times to mix the cells and DNA. Do not vortex.
9. Place the mixture on ice for 10-30 minutes. Do not mix.
10. Heat shock at 42°C for 45 seconds*. Do not mix
    *This heat shock time depends on the strain of bacteria you are using. DH5 alpha uses **45 sec** whereas NEB 10 beta uses **20 sec.**

    ⇒ **Heat shock is the most important step. Heating for too short or too long drastically affects transformation efficiency.**
11. After heat shock, **Immediately** place the mixture on ice for 2 minutes. Do not mix.
12. Add 900µL of ice cooled SOB or SOC media to the tube.
13. Place tube at 37°C for 60 minutes, **in a shaker**! Shake at 150 rpm.

    ⇒ **Shaking aerates the cells and greatly increases transformation efficiency. If you don't shake the cells, your transformation may fail.**
14. Warm plates in incubator to 37°C.
15. Centrifuge tubes on max for 30 sec, decant liquid by gently shaking, leaving 50-100µL left.
16. Pipette vigorously to resuspend the cells.
17. Spread 50-100µL of the cells onto the appropriate antibiotic containing plates.

18. Incubate at 37°C for 12-16 hours, or until colonies are a decent size. Growth time varies with plasmid copy number, overexpression of protein, etc...

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End Chemical Transformation ∅∅∅∅∅

∅∅∅∅∅ **Cleaning Silica Spin Columns for Reuse**

This protocol is based on a protocol by Tagliavia M., Nicosia A., Gianguzza F. 2009. Complete decontamination and regeneration of DNA purification silica columns. *Analytical Biochemistry* 385: 182-183.
This document is version 1.0. Last updated: 1/21/2013.

Solutions
Sterile ddH2O (2.25mL per column)
1N NaOH/0.15% (v/v) Triton X-100 (1.5mL per column)
1.5N HCL/0.15% (v/v) Triton X-100 (.75mL per column)

Materials
1000ul pipettes
Used Silica Columns

You will also need access to a
Centrifuge

Procedure

1. Add 750ul of 1N NaOH/0.15% (v/v) Triton X-100 to each column. Let the columns sit for 5 minutes. Then centrifuge the columns for 1 minute at 13000rpm. Discard the flow-through.
2. Add 750ul of 1.5N HCL/0.15% (v/v) Triton X-100 to each column. Let the columns sit for 30 minutes. Then centrifuge the columns for 1 minute at 13000rpm. Discard the flow-through.
3. Add 750ul of 1N NaOH/0.15% (v/v) Triton X-100 to each column. Let the columns sit for 5 minutes. Then centrifuge the columns for 1 minute at 13000rpm. Discard the flow-through.
4. Add 750ul of sterile ddH2O to each column. Then centrifuge the columns for 1 minute at 13000rpm. Discard the flow-through.
5. Add 750ul of sterile ddH2O to each column. Then centrifuge the columns for 1 minute at 13000rpm.

Discard the flow-through.

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End Cleaning Silica Spin Columns for Reuse ∅∅∅∅∅

∅∅∅∅∅ **Colony PCR**
This protocol is based on a protocol by Knight.
This document is version 1.0. Last updated: 1/21/2013.

Colony PCR is a fast and efficient method of screening ligated colonies for properly ligated inserts. As many ligations rely on the random chance of a part's proper ligation, it becomes important to screen colonies bearing ligated plasmids for these proper clones. Colony PCR suspends raw bacterial colonies in water or LB, then adds the solution itself to the PCR mixture; the high temperature of the PCR reaction will lyse the cells and free the DNA. While it is possible to screen by growing overnight, digesting, and running a subsequent gel, this method takes far more time and resource, though can be more accurate.

It is important that screened colonies are first replicated for downstream reactions. Obtain a plate containing the appropriate antibiotics and label it for the colonies you intend to screen. This will be your "index" plate. You can fit 25 or so colonies on a plate comfortably.

Solutions
taq DNA polymerase
10mM dNTP solutions
Forward, reverse primer solution
diH2O or ddH2O
Bacterial colonies (on plate)

Materials
10, 50µL pipette
PCR tubes (2 per sample)
Colony picker (toothpicks work well)
LB plate with correct antibiotics

You will also need access to a
PCR machine

Procedure

1. Generate a PCR master mix by adding these reagents to a 1.5mL centrifuge tube. This reaction is calculated per 20µL reaction. If you are running multiple reactions, multiply accordingly.

   ⇒ Remember to run a control lane containing a plasmid known to PCR amplify properly. If the assay fails, it will determine whether the colonies or PCR setup itself is at fault.
   2µL 10X taq reaction buffer
   0.4µL dNTP solution (10mM)
   1.0µL Forward primer solution (10µM stock)
   1.0µL Reverse primer solution (10µM stock)
   0.8µL MgSO4 ONLY if fragment to be amplified is larger than 2kbp.
   0.5 units taq polymerase (stock polymerase is 5 units per µL - dilute accordingly)
   ⇒ 2.5 units per 100µL is a good rule of thumb
   diH2O or ddH2O to 20µL final volume

2. Add 20µL H2O to PCR tubes. One tube should exist for every colony screen desired.
   ⇒ This step is vital for the reaction, you must put the colonies in water and transfer the water to the pcr master mix. Any other way will result in massive contamination. We've done the experiments, though you are free to try yourself. :-)
3. Label an "index" plate with spots for colonies you intend to screen. This is important, as any "correct" colonies must be replicated for use in downstream reactions.
4. Using a sterile colony picker (toothpick), pick a colony and suspend it in 20µL H2O by swirling the toothpick.
5. Stab the index plate (1 poke will do!) with the toothpick after suspending the colony in H2O.
6. Add 18µL PCR master mix to a second set of tubes for your samples.
7. Add 1µL colony template solution to each tube. Mix by pipetting up and down.
8. Design a program for your PCR reaction. If using VF2 and VR primers, run the samples on the following PCR program:
   ⇒ Alternatively, use the PCR or similar program already entered into the PCR machine. The current standard program is "VFTAQ".

   A 95°C for 15 mins - breaks open bacterial cells
   B 94°C for 20 seconds
   C 55°C for 20 seconds
   D 68°C for **1 minute per kb expected fragment size**

   Repeat B-D 30 or so times.

   E 68°C for 20 minutes
   F 4°C indefinitely

View the results of the reaction using the gel electrophoresis protocol.

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End Colony PCR ∅∅∅∅∅

∅∅∅∅∅ **DNA Gel Electrophoresis**
Original protocol by Mike Ferguson, background from a previous MSBT protocol.
This document is version 1.0. Last updated: 1/21/2013.

Gel electrophoresis functions as a method of separating DNA strands based on length. From this assessment we can glean a surprisingly varied amount of information-everything from determining if a PCR reaction proceeded correctly to finding mutations. Electrophoresis capitalizes on the negatively charged nature of DNA (or RNA) molecules as well their steric interactions with crosslinked polymers, which vary based on their size. Genetic material is set in a polymer gel made of agarose and pulled towards an electrode with an electric current; larger segments experience more interactions with the agarose polymer and hence move slower than larger ones. Once finished, “bands” are resolved on the material which can be compared by length; their actual length in basepairs can be roughly extrapolated from the gel with a logarithmic scale.

This protocol is involves the compound ethidium bromide which is an intercalating agent-that is, it can fit between the base pairs in a double helix; as a useful side effect, it can fluoresce when exposed to certain wavelengths of ultraviolet light and hence functions as an indispensable means of viewing the DNA. “Laboratory folklore” holds this compound as an extreme toxin, as it is capable of introducing frameshift mutations in genetic material in vitro by virtue of its shape and binding affinity. Tests on higher eukaryotes have the compound to be somewhat toxic; its toxicity, however, has been shown to exist by the virtue of its metabolites and not the chemical itself.

It should hence be noted that while ethidium bromide is not the terrifying poison it has been made out to be, neither is it free of danger and should be handled with care.

Compounds
Agarose
1X TAE, TBE, or SB buffer
Ethidium bromide
Loading dye
Your favorite DNA solution
DNA ladder

Materials
EtBr gel beaker
Weighing boat
Gel holder
Gel cast
Pipettor, 10 or 20µL pipette tips

You will also need access to a
Scale
Microwave
Gel Viewer
Ethidium bromide disposal container

Procedure

1. Weigh out the correct amount of Agarose for your gel. Always make 100mL of gel at a time, so you should be measuring out somewhere between .5g - 4g of agarose. 1.5g (1.5%) - 2g (2%) is recommended for most applications. Choose what you like best. Note, accuracy in this protocol is not super important, so don't fret over measuring errors.
2. Measure out 50mL/gel of either TBE,TAE, or SB buffer in your designated Ethidium Bromide gel making beaker. A 500mL beaker works well.
3. Add the agarose to the beaker and swirl it around for a few seconds.
4. Place the mixture in the microwave for about 1 min 15 sec - 1 min 30 sec on high. Swirl it to mix up any agarose remaining undissolved and heat it again briefly for 15 - 30 sec. The goal is to heat the solution so all the agarose is dissolved (no white powder visible) and there are no or minimal amounts of bubbles. Keep an eye on it to make sure it doesn't boil over.
5. Add 5µL/gel Ethidium Bromide or substitute to the heated gel solution. Swirl to mix. If there are still a decent amount of bubbles in the gel, heat the mixture until they go away. You may have to fiddle with it. Heating in 10, 15, or 20 sec intervals depending on how many bubbles there are is usually ideal.
6. Pour your gel into your gel holder. If there are bubbles, use a pipette tip or the well casts to move the bubbles to the bottom edge of your gel.
7. Place your well casts into the gel. It sucks if you forget to do this step cause you have to remelt your gel :p
8. Wait for your gel to dry. This usually takes around 20-30 minutes.

   ⇒ Start preparing your samples (See step 12) while your gel dries.

9. Once dry, remove the well casts by lifting them straight out. You may have to wiggle them a bit at first to loosen them. If there are chunks of agarose in the wells afterwards, you can use a pipette tip to remove them.
10. Position your gel with the top (side with the wells) towards the black negative electrode. It sucks when you mess this up too. :p
11. Pour in 1x TBE, TAE, or SB Buffer into the gel box until it just covers the top of the gel.
12. Prepare your DNA samples by adding 5µL 6x loading dye per 25µL of DNA.
13. Add at least 10 - 15µL of DNA into the well without it overflowing. 10µL is usually good. Don't forget your ladder! Instructions for making the DNA ladder can usually be found online or in the manual that comes with the ladder. Sometimes, making the ladder in bulk messes up the concentration for some reason. Perhaps pipette error (who knows when our pipettes were last calibrated...). You might have to make it in small aliquots.

⇒ If your hand is shaking a lot, rest your other hand on the side of the pipette to stabilize yourself. press the plunger down **slowly** and smoothly. 10µL tips are a lot easier to load with than 20µL tips.

14. Run the gel. 135 volts for 30 - 45 min is usually good. You can vary it how you see fit. Beware, you can run it too fast! Your bands will smile (be curved) and distances will be messed up.
15. Once finished, visualize on the viewer. How exactly to operate the viewer will vary slightly, but the important thing to know is you can change the zoom and contrast on the gel viewer (usually with black cylinders near camera with white numbers on them). You can also mess with exposure time. These things use UV light, so be careful!!!!!!!!!! Do not expose your skin, eyes or any part of you to the UV light. You will be burned severely within seconds.
16. Print or upload the gel image to a USB stick. The digital images are recommended.

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End DNA Gel Electrophoresis ∅∅∅∅∅

∅∅∅∅∅ **Making LB Plates**

Original protocol by Mike Ferguson, Background adapted from a previous MSBT protocol.
This document is version 1.0. Last updated: 1/21/2013.

This protocol will generate plates containing LB nutrient broth to grow bacteria, agar, and your choice of antibiotic. While generating the initial LB is does not require strong sterile technique as it will be sterilized in the autoclave, a strongly bacteria-free environment will be necessary for pouring the plates.

After agar LB is autoclaved, the liquid should be handled very carefully-any introduced bacterial will contaminate the stock.

This protocol will produce ~25-35 plates. The external protocol on *autoclaving* will be used in addition to this one.

Compounds
dH2O
LB broth powder
Agar powder
Your favorite antibiotic

Materials
1L Erlenmeyer flask
Weigh boats
Petri dishes
Permanent markers

You will also need access to an
Autoclave
Refrigerator

Scale

External protocols
Autoclaving

Procedure

1. Fill a 1L Erlenmeyer flask to ~600mL with dH2O.
2. Measure out 15g of agar powder and 25g of LB powder. Add these to the 1L flask, swirl to mix the solution well or use a stir bar.
3. Fill the 1L flask up to the 1000mL mark with dH2O. Make sure it is mixed completely.
4. Aliquot the media into 500mL jars. Autoclave the media.
5. Let the autoclaved LB agar solution sit on a counter until cool enough to hold without a glove or sit it in a water bath at ~55°C.
6. Add your antibiotic if you need to. Mix well. If you add your antibiotic too soon, when the media is still hot, the antibiotic will be denatured and won't work.
7. Ensuring the work area is sterile, remove the sterile Petri dishes from the bag and put a few of them in a line. Pour plates to approximately ½ full. Put tops back on.
8. Let the plates dry. If using ampicillin, cover the plates with aluminum foil. Ampicillin is light sensitive.
9. Store with the tops face down in a fridge to avoid having water condense on to the media.

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End Making LB Plates ∅∅∅∅∅
∅∅∅∅∅ **Plasmid DNA Purification Using the QIAprep Spin Miniprep Kit and a Microcentrifuge**
Copied from Qiagen Handbook on 1/21/2013. You probably have this handbook laying around in the lab somewhere.

This protocol is designed for purification of up to 20 µg of high-copy plasmid DNA from 1–5 ml overnight cultures of E. coli in LB (Luria-Bertani) medium. For purification of low-copy plasmids and cosmids, large plasmids (>10 kb), and DNA prepared using other methods, refer to the recommendations on page 44.

Please read "Important Notes" on pages 15–21 before starting.
Note: All protocol steps should be carried out at room temperature.

Procedure

1. **Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a microcentrifuge tube.**

   Ensure that RNase A has been added to Buffer P1. No cell clumps should be visible after resuspension of the pellet.

   If LyseBlue reagent has been added to Buffer P1, vigorously shake the buffer bottle to ensure LyseBlue particles are completely dissolved. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

2. **Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4–6 times.**

   Mix gently by inverting the tube. Do not vortex, as this will result in shearing of genomic DNA. If necessary, continue inverting the tube until the solution becomes viscous and slightly clear. Do not allow the lysis reaction to proceed for more than 5 min.

If LyseBlue has been added to Buffer P1 the cell suspension will turn blue after addi- tion of Buffer P2. Mixing should result in a homogeneously colored suspension. If the suspension contains localized colorless regions or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved.

3. **Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times.**

   To avoid localized precipitation, mix the solution thoroughly, immediately after addition of Buffer N3. Large culture volumes (e.g. ≥5 ml) may require inverting up to 10 times. The solution should become cloudy.

   If LyseBlue reagent has been used, the suspension should be mixed until all trace of blue has gone and the suspension is colorless. A homogeneous colorless sus- pension indicates that the SDS has been effectively precipitated.

4. **Centrifuge for 10 min at 13,000 rpm (~17,900 x g) in a table-top microcentrifuge.**

   A compact white pellet will form.

5. **Apply the supernatants from step 4 to the QIAprep spin column by decanting or pipetting.**
6. **Centrifuge for 30–60 s. Discard the flow-through.**
7. **Recommended: Wash the QIAprep spin column by adding 0.5 ml Buffer PB and centrifuging for 30–60 s. Discard the flow-through.**

   This step is necessary to remove trace nuclease activity when using endA+ strains such as the JM series, HB101 and its derivatives, or any wild-type strain, which have high levels of nuclease activity or high carbohydrate content. Host strains such as XL-1 Blue and DH5αTM do not require this additional wash step.

8. **Wash QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging for 30–60 s.**
9. **Discard the flow-through, and centrifuge for an additional 1 min to remove residual wash buffer.**

   Important: Residual wash buffer will not be completely removed unless the flow-through is discarded before this additional centrifugation. Residual ethanol from Buffer PE may inhibit subsequent enzymatic reactions.

10. **Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of each QIAprep spin column, let stand for 1 min, and centrifuge for 1 min.**

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End Plasmid DNA Purification Using the QIAprep Spin Miniprep Kit and a Microcentrifuge ∅∅∅∅∅

∅∅∅∅∅ **QIAquick Gel Extraction Kit Protocol using a microcentrifuge**
copied from QIAquick Spin Handbook on 1/21/2013

This protocol is designed to extract and purify DNA of 70 bp to 10 kb from standard or low-melt agarose gels in TAE or TBE buffer. Up to 400 mg agarose can be processed per spin column. This kit can also be used for DNA cleanup from enzymatic reactions (see page 8). For DNA cleanup from enzymatic reactions using this protocol, add 3 volumes of Buffer QG and 1 volume of isopropanol to the reaction, mix, and proceed with step 6 of the protocol. Alternatively, use the MinElute Reaction Cleanup Kit.

Important points before starting

- The yellow color of Buffer QG indicates a pH ≤7.5.
- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at 17,900 x g (13,000 rpm) in a conventional table-top microcentrifuge at room temperature.

Procedure

1. **Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.**

   Minimize the size of the gel slice by removing extra agarose.

2. **Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl).**

   For example, add 300 µl of Buffer QG to each 100 mg of gel. For >2% agarose gels, add 6 volumes of Buffer QG. The maximum amount of gel slice per QIAquick column is 400 mg; for gel slices >400 mg use more than one QIAquick column.

3. **Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2–3 min during the incubation.**

   IMPORTANT: Solubilize agarose completely. For >2% gels, increase incubation time.

4. **After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose).**

   If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.

   The adsorption of DNA to the QIAquick membrane is efficient only at pH ≤7.5. Buffer QG contains a pH indicator which is yellow at pH ≤7.5 and orange or violet at higher pH, allowing easy determination of the optimal pH for DNA binding.

5. **Add 1 gel volume of isopropanol to the sample and mix.**

   For example, if the agarose gel slice is 100 mg, add 100 µl isopropanol. This step increases the yield of DNA fragments <500 bp and >4 kb. For DNA fragments between 500 bp and 4 kb, addition of isopropanol has no effect on yield. Do not centrifuge the sample at this stage.

6. **Place a QIAquick spin column in a provided 2 ml collection tube.**
7. **To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min.**

   The maximum volume of the column reservoir is 800 µl. For sample volumes of more than 800 µl, simply load and spin again.

8. **Discard flow-through and place QIAquick column back in the same collection tube.**

   Collection tubes are reused to reduce plastic waste.

9. **Recommended: Add 0.5 ml of Buffer QG to QIAquick column and centrifuge for 1 min.**

   This step will remove all traces of agarose. It is only required when the DNA will subsequently be used for direct sequencing, in vitro transcription, or microinjection.

10. **To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min.**
    Note: If the DNA will be used for salt-sensitive applications, such as blunt-end ligation and direct sequencing, let the column stand 2–5 min after addition of Buffer PE, before centrifuging.

11. **Discard the flow-through and centrifuge the QIAquick column for an additional 1 min at 17,900 x g (13,000 rpm).**

    IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.

12. **Place QIAquick column into a clean 1.5 ml microcentrifuge tube.**
13. **To elute DNA, add 50 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand**

for 1 min, and then centrifuge for 1 min.

IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl.

Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.

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End QIAquick Gel Extraction Kit Protocol using a microcentrifuge∅∅∅∅∅

∅∅∅∅∅ **QIAquick PCR Purification Kit Protocol using a microcentrifuge**
copied from QIAquick spin handbook on 1/21/2013

This protocol is designed to purify single- or double-stranded DNA fragments from PCR and other enzymatic reactions (see page 8). For cleanup of other enzymatic reactions, follow the protocol as described for PCR samples or use the MinElute Reaction Cleanup Kit. Fragments ranging from 100 bp to 10 kb are purified from primers, nucleotides, polymerases, and salts using QIAquick spin columns in a microcentrifuge.

I**mportant points before starting**

- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at 17,900 x g (13,000 rpm) in a conventional tabletop microcentrifuge at room temperature.
- Add 1:250 volume pH indicator I to Buffer PB (i.e., add 120 µl pH indicator I to 30 ml Buffer PB or add 600 µl pH indicator I to 150 ml Buffer PB). The yellow color of Buffer PB with pH indicator I indicates a pH of □7.5.
- Add pH indicator I to entire buffer contents. Do not add pH indicator I to buffer aliquots.
- If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without the addition of pH indicator I.

⇒ MSBT note: You can use this protocol with Buffer PB that doesn't contain the pH indicator

Procedure

1. **Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix. It is not necessary to remove mineral oil or kerosene.**

For example, add 500 µl of Buffer PB to 100 µl PCR sample (not including oil).

2. **If pH indicator I has beein added to Buffer PB, check that the color of the mixture is yellow.**

   If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.

3. **Place a QIAquick spin column in a provided 2 ml collection tube.**
4. **To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s.**
5. **Discard flow-through. Place the QIAquick column back into the same tube.**

   Collection tubes are re-used to reduce plastic waste.

6. **To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s.**
7. **Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min.**

   IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.

8. **Place QIAquick column in a clean 1.5 ml microcentrifuge tube.**
9. **To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.**

   IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl elution buffer.

   Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.

Return to Protocols

End QIAquick PCR Purification Kit Protocol using a microcentrifuge∅∅∅∅∅

∅∅∅∅∅ **Removing Biobricks from the Wells**

This protocol is based on a protocol on the IGEM 2011 website.
This document is version 1.0. Last updated 1/21/2013.

This procedure will remove BioBricks from the parts wells. The DNA in these wells is in a special dehydrated state that must be redissolved in solution before it can be used.

The protocol will require ddH2O, or ultrapure water. Water of this nature is usually available in commercial stocks or from giant, expensive looking water purification machines. Even if sterile, standard dH2O or diH2O can contain DNA digesting enzymes or *DNAses* left over from lysed bacteria and viruses capable of destroying DNA samples. Since these samples are being stored for long periods of time, the presence of even partially functioning DNAses can have catastrophic results.

The protocol will remove and rehydrate the DNA, which is in the form of a part with biobrick components readily transformable as a vector; transformation will be called by an external protocol.

Compounds
DNA plate stocks ddH2O or ultrapure H2O

Materials
1.5mL centrifuge tube (sterile, chilled to 4°C) 5-50µL pipette

You will also need access to a: Freezer at -20°C

External protocols
Quick Transformation protocol -or-
Heat shock protocol -or-
Electroporation protocol

Procedure

1. Determine the location of the well containing your part. Be careful!
2. Punch through the foil covering the well with a 5-50 pipette.
3. Add 10µL ddH2O or ultrapure water to the well. Mix by repeatedly intaking and expelling the solution from your pipette, about 3 times.
4. Wait 5 minutes for the DNA to resuspend.
5. Transfer the entire contents of the well to a new 1.5mL centrifuge tube with a 5-50 pipette.
6. Use the solution to transform the bacteria, or store the stocks in a freezer at -20°C

⇒ Proceed to any transformation protocol to generate stocks of the DNA.

Return to Protocols

End Removing Biobricks from the Wells ∅∅∅∅∅

∅∅∅∅∅ **Restriction Enzyme Digest**
This protocol is a consensus protocol between protocols on NEB and OpenWetware.
This document is version 1.0. Last updated: 1/21/2013.

Restriction enzymes are enzyme capable of cutting DNA at specific locations. Often used by bacteria as a defense against viral genes, they are readily useful in biology and genetic engineering as tools for modifying custom DNA sequences.
Stock enzymes must be kept at very cool temperatures or they will denature. It is imperative the enzyme stocks are kept in ice when removed from the freezer.
We currently use NEB Buffers. These buffers vary greatly in their ability to support enzymes, especially with multiple digests. The following table demonstrates buffers to be used with standard BioBrick enzymes (values are %×100):

NEBuffer	1	2	3	4
EcoRI	1.0	1.0	1.0	1.0
EcoRI-HF	0.1	1.0	0.0	1.0
XbaI	0.0	1.0	0.75	1.0
SpeI	0.75	1.0	0.25	1.0
PstI	0.75	0.75	1.0	0.5

⇒ When in doubt, use NEBuffer 2.

This protocol will vary depending on the nature of the digest. This reaction mixture will produce 50µL total DNA; if the DNA to be used is dilute, double it to 100µL! Do not double enzyme concentration!

Compounds.
DNA solution
Restriction enzyme(s)
10X NEBuffer
diH2O
100X BSA solution

Materials
Pipettes
PCR tubes
ICE

You will also need access to a:
37° heat source
Vortexer

Procedure
⇒ This can be changed proportionally.

1. Add 10-20µL DNA solution to the PCR tube.
2. Design a master mix for each pair of enzymes to be used. For example, if cutting 2 parts XbaI and PstI and 2 parts with EcoRI and PstI, design a mix ~3 volumes large for the former solution and ~2 volumes large for the latter.

   Add 5µL 10X NEBuffer, 0.5µL 100X BSA, and dH2O to 50µL per part to be digested to form a master mix. \
   ⇒Add enough ingredients for 1-3 extra digests to account for pipette error.
   ⇒Double these volumes (100µL final) when calculating master mixes if DNA is known to be dilute.

3. Add 1µL enzyme(s) per part to be digested to the master mix. (must be added last)
   ⇒ Carefully touch the tip if your pipette to the surface of the glycerol (don't plunge the pipette in!) and withdraw the volume; wait a second for the liquid to fully flow into the pipette, then add to your reaction mixture.
4. Aliquot the mixture to respective tubes containing DNA. Flick to mix.
5. Incubate at 37°C for 2-3 hours, then heat inactivate at 80° C for 15 minutes.

⇒ If a vector is being digested, add a phosphatase to decrease its self-ligation frequency.

1. Add 1µL phosphatase to your vector digestion. Do not add to both vector and insert, as this will destroy their ability to ligate together! If using Antarctic Phosphatase, unless Zn has already been added to the previous buffers, also add 6 uL of 10X Antarctic Phosphatase Buffer and 3uL more of water.
2. Incubate 37° C for 60 min.
3. If using CIP (Calf Intestinal Phosphatase), you must PCR purify the samples. If using Antarctic Phosphatase, you can heat inactivate at 70° C for 5 minutes.

⇒ Proceed to the DNA Purification (PCR/Digest) protocol.

Return to Protocols

End Restriction Enzyme Digest ∅∅∅∅∅

ØØØØØ T4 Ligase Protocol
This protocol based on a protocol on openwetware somewhere.
This document is version 1.0. Last updated 1/21/2013.

T4 allows for the fusion of free DNA ends provided they are complementary or blunt and phosphorylated. Ligase relies on ATP, which is provided in the buffer at a concentration of 10mM.

A series of ligations will be set up, as ligation effectiveness is unreliable and heavily dependent on complex factors including DNA length, concentration, etc. DNA stocks should ideally be purified after a digestion, but ligations can still work even if fragments contain NEBuffer or residual (inactivated) restriction enzyme.

The total volume in the PCR tube should come to 10µL. Any volume left below 10µL should be filled with ddH2O.

*Do not attempt to ligate fragments amplified by PCR unless you are confident they are clean and possess minimal background!

Compounds
10x T4 ligase buffer
T4 ligase (in glycerol)
ddH2O or autoclaved diH2O
Linearized vector (in EB or ddH2O)
Linearized insert(s) (in EB or ddH2O)

Materials
10µL pipette, tips
PCR tubes

External protocols:
Heat shock transformation

NOTE on ligase units!: NEB uses different units than other companies. They use **CELU** (Cohesive End Ligation

Units) units. Other companies use **Weiss** units.

1 Weiss unit = ~67 CELU units

For single insert construction, set up the following ligation mixture:

1μL Plasmid
3μL Insert
1μL Ligase Buffer
1μL T4 Ligase Enzyme (**diluted in 50% glycerol to 100 CELU units per μL**)
4μL sterile water

For double insert/3A construction, set the following ligation mixture:

1μL Plasmid
3μL Insert 1
3μL Insert 2
1μL Ligase Buffer
1μL T4 Ligase Enzyme (**diluted in 50% glycerol to 100 CELU units per μL**)
1μL sterile water

Mixture Incubation
If sticky ends are used, incubate at room temp. for 10 min or 4°C (fridge) overnight.
If blunt ends are being ligated, incubate at 15-20°C for 16-24 hours.

⇒Proceed to transform ligation mixtures using the entire volume of the mixture.

Return to Protocols

End T4 Ligase Protocol ∅∅∅∅∅

∅∅∅∅∅ **Vent PCR**
This protocol is based on a protocol by NEB.
This document is version 1.0. Last updated: 1/21/2013.

PCR is a rapid method of amplifying low concentrations of DNA into high concentrations capable of being viewed or used. This protocol will utilize NEB's VentR polymerase, a fairly high accuracy polymerase. VentR is should only be used for cloning. For routine PCR, please use taq. For an Overview, click here.

Enzyme concentration is 2 units/µL; generally 1 unit per 100µ L is ideal.

Solutions
ThermoPOL reaction buffer
100mM MgSO4 solution (optional) dNTP solution
Forward primer solution, concentrated Reverse primer solution, concentrated VentR polymerase solution
ddH2O
Template solution

Materials
10, 50µL pipette
PCR tubes
1.5mL centrifuge tubes.

You will also need access to a
PCR machine
Tabletop quick centrifuge

Procedure

1. Generate a PCR mix by adding these reagents to a 1.5mL centrifuge tube. This reaction is calculated per 50 µL reaction-if you are running multiple reactions, multiply in accordance with your samples!

5µL 10X ThermoPol buffer
1.0µL dNTP solution (10mM)
0.5-2.5µL Forward primer solution (10µM stock)
0.5-2.5µL Reverse primer solution (10µ M stock)
2µL 100mM MgSO4 **ONLY if fragment to be amplified is larger than 2kbp.**
1.25µL 0.4 units/µ L VentR polymerase (1µL polymerase in 4µL 1X reaction buffer)
NOTE: ⇒ 1 unit per 100µL is a good rule of thumb-this will add 0.5 units to the 50µL reaction. diH2O or ddH2O to 50 µL final volume
⇒ Pipetting tiny amounts of polymerase can be impossible. Dilute 1µL polymerase in 4µL 1X ThermoPol buffer in a separate tube, then add diluted polymerase to reaction (for 50µL reaction, 2.5µL @ 0.4 units/µL polymerase).

2. Properly divide the solution among your samples.

3. Add 0.5-1.0µL template solution to each sample. Mix by uptaking and expelling liquid with your pipette.

4. Centrifuge the tubes in a tabletop quick centrifuge for 20 seconds.

5. Design a program for your PCR reaction:

A. 95°C for 2-5 mins (dependent on length of template)
B. 95°C for 15-30 seconds
C. 55-65°C for 15-30 seconds
NOTE: ⇒ Dependent on Tm of primers! For Vf2/Vr, 60°C)
D. 72°C for **60 seconds per kb expected fragment size**
E. 72°C for 5 minutes
F. 4°C indefinitely

Repeat B→ D 30 times.

Return to Protocols

End Vent PCR∅∅∅∅∅

Protein purification

FimE and HbiF purification

1. Inoculate 100 mL LB medium (Kan100) and grow **overnight** at 37°C @ 260 rpm
2. Transfer 25 mL culture to 1 Liter PEM medium (Kan100) and further incubate at 37°C on the shaker (260 rpm)
3. Measure the O.D. until your cells reach mid-log-phase ($3.0 \leq OD \geq 4.0$)
4. Take a sample, and then cool down the incubator to 20 *C (while shaking the cells) for approximately 1 h. After that induce protein expression with IPTG (c(final) = 0.1mM) and further incubate the cells **overnight** (20 °C, 260 rpm)
5. Take a sample, and then harvest your cells (centrifuge at 7000 rpm for **10 minutes** in 1 L bottles at 4 °C)

6. **FREEZE O/N**
7. Re-suspend the pellet of 1 L of cell culture in 50 mL enriched lysis buffer on ice:

   · Lysis-buffer = 50mM Tris, 400mM NaCl, pH8.0, 10%glycerol

   · Enriched = add to 50 mL lysis buffer: 1 tablets of protease inhibitors (complete, Roche), 3mM 2-Mercaptoethanol, 0.5mg/ml lysozyme (scoop tip!), 200 uL DNase(1 mg/ml stock))

1. Homogenize the suspension with an Ultratorax and then let it sit on ice for **10 minutes**.
2. Lyse the cells using a french press (high pressure, ~ 1200 psi, high pressure mode; make sure that the pressure does not go below 1000 during lysis).
3. Take a sample of the lysate, and then centrifuge the lysate at 15,000 rpm at 4°C for **1 hours.**
4. In the meanwhile, equilibrate Nickel column with 5 x column volume of water, then 5 x column volumes of lysis buffer in the cold room (buffer needs to be cold!).

11. After centrifugation, take out supernatant (SN) of the spun lysate carefully. Take a sample of the supernatant. Pass the SN through a 0.45 um (maybe 0.8 um) filter. Check the solution for aggregates before you proceed.

1. Slowly pass SN through the nickel column. Take a sample of the flow-through. Keep the flow-through!
2. Wash the loaded column with 10 column volumes (50 mL) of:

   Lysisbuffer (containing 30 mM Imidazole)
   Take a sample of the first 5 mL of the wash solution.

1. Elute protein from column with 1 column volume of lysis buffer (containing 250 mM Imidazole) Take a sample of the elution.

   Then add 1 tube of ULP1 to the eluted solution and digest **O/N**.

1. After o/n in-solution digestion, take a sample of the solution.
2. Pass the solution through the Nickel column and collect flow through. take a sample of the flow through.
3. On the side, elute rest of protein in a separate container with lysis buffer (containing 250 mM Imidazole) Take a sample of the elution.
4. Pass previously eluted protein through a 5 mL Cation Affinity column (SP FF).
5. Then elute protein from the SP FF column with a linear gradient of 0-0.8 M NaCl (use buffer A (with 0.5 mM EDTA) + 1 M NaCl for buffer B, then run 0-80% gradient). Take a sample of each fraction that contain a protein.
6. Pool the fractions that contain pure protein and dialyze 2 x for 1 h against 1 L, then 1 x o/n against 3 L of HNX (50 mM Tris, 100 mM NaCl, containing 0.5 mM EDTA). Spy contains a

lot of methionine that can be oxidized by traces of metals. Take a sample.

7. Concentrate dialyzed sample, aliquot and freeze in liquid N2. Take a sample of the concentrated solution.

Buffers to prepare. Apart from the dialysis buffers, filter all the other ones! Degasing is not necessary!

Taking a sample:

Cells: take 1.5 mL of cells and centrifuge them down (8000 rpm for 6 min). Then re-suspend the pellet in 100 uL 50 mM NaPi, pH 8. Finally, add 25 uL reducing loading buffer.

Otherwise: take 100 uL and add 25 uL reducing loading buffer