Sterile Technique and Pipetting

Introduction

The most important skill you need in the molecular lab is and technique and precision. Technique will not only dictate the success of your work, it will determine how much work you need to do. One small mistake as a result of bad technique may mean you have to start over entirely, or spend a great deal of time troubleshooting, or have to purchase new reagents. Even worse, contamination may provide a misleading result, such as thinking CBB is present in a field when it is not, costing time and money.

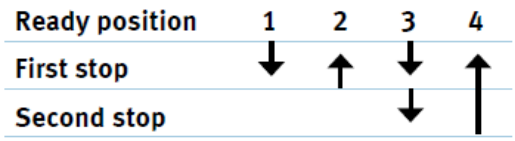
A clean work environment

The molecular lab should be clean, and checked **at least** once a week.

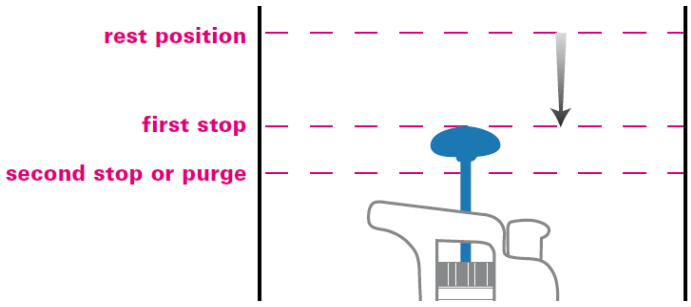
- No unauthorized persons or activities in the lab
- No dirt, dust, excessive moisture, live insects or animals, anywhere in the lab
- No direct openings to the outside
- All equipment should be organized and labeled. No clutter or papers on countertops.
- Wearing lab coats is good practice for reducing contamination and keeping the lab clean.
- Air filters in AC units and flow hoods should be replaced if dirty.
- Labeling <u>everything</u> is vital, with as much information as possible (names, dates, descriptions). If anything is found un-labeled, it should be thrown away immediately.
- No equipment is to be removed

Operating a pipette

Typically as follows:

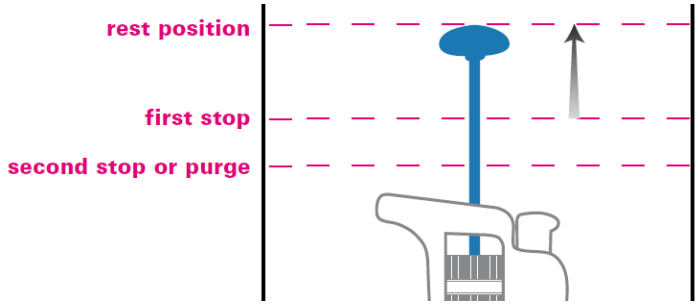


1. Firmly but gently press the pipet at the top button. Do not use too much force. Press the operating button to the first stop.

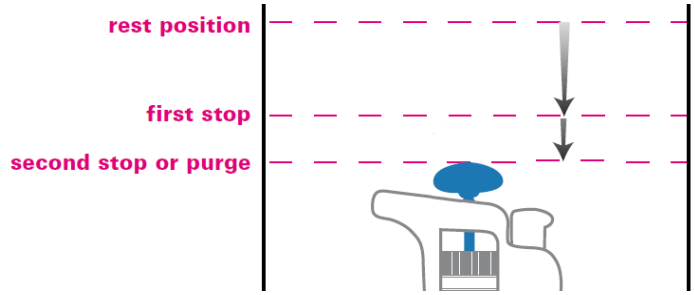


2. Holding the top vertical with the button held at the first stop, dip the tip into the solution. **Slowly** release the operating button, drawing the liquid into the tip. Withdraw the tip from the liquid without touching the sides of the vessel. **Never** turn the pipette

horizontal while there is liquid in the tip, or allow liquid to be sucked into the pipette itself, as this will cause damage to the device.



3. Dispense the liquid into the receiving vessel by **gently** pressing the operating button to the first stop, wait a half second, then press the button to the second stop. Remove the tip from the vessel, sliding it along the wall of the vessel. There should be no remaining liquid in the tip, and the operating button should be back in operating position



4. Pipet tips are single use. Do not shoot the tip off of the pipette violently. Gently release it into a waste bin without causing leftover liquid to be splashed or shot outside of the waste bin.

Keeping your work sterile and contaminant-free

Before beginning, your work station, and gloves, should be wiped down with 10% bleach.

Another important way to minimize contamination, and aid in troubleshooting if something goes wrong, is to make aliquots of stock reagents and keep materials from different project separate. Stock reagents (as opposed to working solutions), DNA extracts and PCR amplicons can be kept in a shared space, but all other reagents and working solutions need to be kept in your own sample box, labeled with your own name, and never used by another scientist.

Data management

Excel and databases

Good record keeping is vital for preventing confusion and wasted efforts, and will help in troubleshooting. Backup files regularly (weekly) on external drives and online.

If the lab is in permanent high use, the manager of the lab should consider using database software to keep track of all data. For the time being, excel will work well for keeping records. Always create a record **before** completing a procedure.

Extract-N-Amp DNA extractions

Spin-down protocol (for beetles)

Keep BSA and the Ex-n-A solution cold, on ice or in a pre-frozen rack.

1. In a PCR tube, crush the beetle body (fresh or removed from 90% ethanol) with a pipette tip
2. Add 40uL of Extraction Solution per crushed beetle sample
3. Add the samples to the thermocycler and run the Ex-n-Amp protocol (96C for 30 minutes)
4. Once completed, add equal volume (40uL, to extract solution) of 3% BSA (30mg/mL =~3%)
5. Vortex thoroughly
6. Spin down the solution in the centrifuge at 10,000 G for 30 seconds
7. Store the upper half (30uL) as your final sample
8. You will typically use 0.5-1.0uL of this final supernatant for PCR

Spin-down protocol (for clean cultures of fungi)

Keep BSA and the Ex-n-A solution cold, on ice or in a pre-frozen rack.

1. In a PCR tube, add about 10uL of fresh fungus tissue (fresh the outside edge of a clean fungal colony on a culture plate) with a flame-sterilized scalpal
2. Add 20uL of Extraction Solution per fungus sample
3. Add the samples to the thermocycler and run the Ex-n-Amp protocol (96C for 30 minutes)
4. Once completed, add equal volume(20uL, to extract solution) of 3% BSA (30mg/mL =~3%, will bind extra stuff)
5. Vortex thoroughly
6. Spin down the solution in the centrifuge at 10,000 G
7. Store the upper half (20uL) as your final sample
8. You will typically use 1.0uL of this final supernatant for PCR

ExTaq PCR

Introduction

A PCR contains many components designed to rapidly copy a very small portion of an organism's genome. The components include:

- **DNA template/extract**: genomic DNA from a target organism, what you produce from a DNA extraction procedure
- **Nucleotides**: Adenine, Cytosine, Thiamine, and Guanina (ACTG) are the building blocks of DNA. The goal is to find a sequence of these nucleotides in a specific part of the genomic DNA.
- **Primers (2):** short fragments of DNA that find a specific region of DNA you want to copy.
- **Taq**: this is a heat-sensitive enzyme which is able to copy DNA.
- **Buffer and water**: Stabilizes the reaction and makes it less concentrated.

Takara ExTaq

Store at -20°C. After it has been thawed, store at 4°C, do not re-freeze.

Diluting primers

Primers arrive lyophilized (dried) unless they are ordered as "Lab Ready." To reconstitute lyophilized primers to create a 100X "stock concentration (100 uM/L)," add PCR water. For example: for 28.5 nM of lyophilized primer, add 285 uL water (10 times more, in uL).

For use in PCR, primers need to be again diluted, this time in aliquots, to a "working concertation." To dilute to make working solution, use 10uL of the "stock concentration (100 uM/L)," add 90 uL of PCR water, to make 10 uM/L "working solution." Working solutions should be kept in person-specific or project-specific boxes.

Order from: https://www.idtdna.com/pages/products/dna-rna/custom-dna-oligos

Running a PCR

Running a PCR is relatively easy. All you need to do is add the below components, and then put it into the thermocycler using the correct conditions.

Typical master mix:

1. In an excel spreadsheet, multiply the amount of each component of a mastermix by how many samples you want to perform a PCR on. There will usually be an example already on the PCR sheet which you can edit.
2. Add all the components together in a single tube, **NOT including DNA extracts**.
3. Take portions of the total mixture, and divide it equally into each tube, typically 24 uL of mastermix, per sample.

4. Add your DNA extract (1uL) to the separate sample tubes already containing mastermix, creating a final volume of 25uL per sample.
5. The samples can now be added to the thermocycler, and ran using the correct conditions.

Cycling conditions:

You will need to check the literature for what cycling conditions are best for your organism and intended gene region. You can check past PCRs in the lab database to see what has been successful in the past.

Running electrophoresis gels

Introduction

There are many uses for gel electrophoresis in a molecular lab, but here the purpose will be to make sure your PCR was successful. Electrophoresis does this by showing you how long DNA fragments are. You should have an idea of how long your PCR amplicon sequence should be. If not, you can search online for scientific articles that have sequenced your organism in question.

Because DNA is negatively charged, it runs towards the positive end (cathode) of the polarized gel rig. Longer fragments move more slowly as they are larger. The DNA ladder included in your gel electrophoresis contains many DNA fragments of different, known sizes, so that you can compare the distances traveled of your PCR product, to those in the ladder.

Preparing Gel

Remember the 50X stock solution of TAE needs to be diluted to a 1X working solution

For the small tray, you need 60mL of 1% gel (0.6g of agarose to 60 ml of 1X TAE buffer). Boil in microwave or hot plate until the agarose is completely dissolved after stirring. Cool down under water, pour into the rig with the well comb already inserted, and wait for about 15 min to solidify before pulling the well comb out.

Preparing SYBR Green

For loading directly to the wells in the gel with your sample

SYBR® Green I Stain can be added directly to the loading buffer at a final "stock concentration" of 1:1000. First prepare a 1:100 dilution of SYBR® Green I Stain in high-quality anhydrous DMSO. The 1:100 dilution can be stored in the freezer and reused.

SYBR Green & Dye mastermix

For one sample, combine 0.5uL of 100x "working concentration" SYBR (labeled 1:100X) and 1uL of 6X Loading Dye. Mix that with 5uL of PCR product

For mastermix, combine 300uL of 100x SYBR and 600uL of Dye.

Loading the Gel

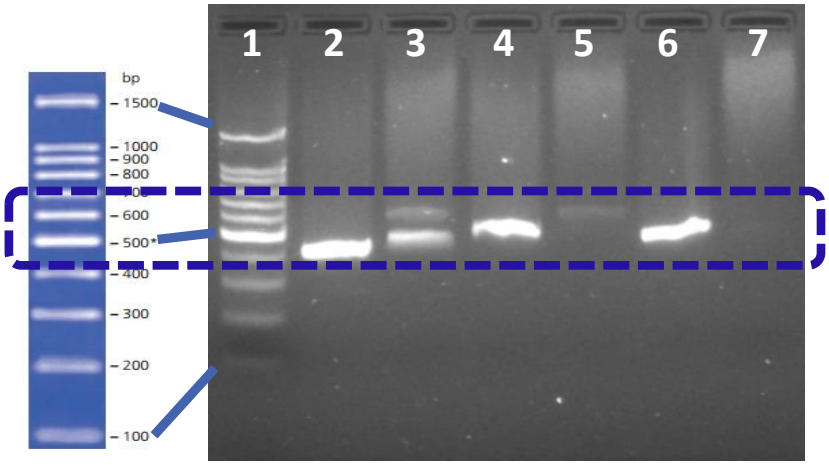
An electrophoresis gel should be completely submerged by 1X TAE buffer. Just enough TAE to cover the top of the gel is best. Then you will add your mixture of SYBR+Dye and PCR product to each well as follows:

Prepare ~1uL droplets of SYBR & Dye mastermix on parafilm. First add 6uL of 100bp DNA ladder and mix by sucking up&down with the pipette. Add the entire droplet to the first well. For

the following wells, combine 5uL of PCR product with the 1uL droplets of SYBR and Dye mastermix.

Connect the gel rig to the power supply, connecting red to black. The DNA will run through the gel to the red end (remember "runs to red"). Run on about 120 Volts for 40-60 minutes. Wait by the gel for the first minute to make sure everything is running correctly.

Photographing the gel



1. DNA ladder
2. ~450 base pair PCR amplicon (DNA fragment)
3. Double bands indicate contamination
4. ~500 base pair PCR amplicon
5. Less concentrated PCR amplicon
6. Positive control
7. Negative control

In an image editor such as MS paint or (ideally) IrfanView, label each lane with their corresponding PCR number and ladder type. Also edit the image filename to "date_PCR NNNN-NNNN." For example, "2016-7-8_PCR 25-38." Store the gel photo in a folder with lab records and remember to backup files regularly.

PCR Cleanup

Introduction

Once the presence of the PCR amplicons has been confirmed by gel electrophoresis, the PCR product needs to be cleaned prior to sequencing. The cleaning removes leftover molecules which could interfere with sequencing. This can also be done by the sequencing facility at little-cost. **CIC will outsource this task to our sequencing facility, Macrogen, for the time being.**

Keep Exosap on ice and don't keep out of freezer longer than necessary. This product doesn't freeze so it is always ready to use straight out of the freezer.

Cleanup for Sanger sequencing

1) Mix in a PCR tube for each sample:
   - .5 uL PCR water
   - 1 uL Exosap
   - 2 uL PCR product
2) After spinning down, run ExoSap program on thermocycler (37C for 15 min, and 80C for 15 min).
3) divide half (3 uL) per 1.5 uL tube and the remaining half into a second 1.5 uL tube. These will be your forward and reverse submissions for sequencing.

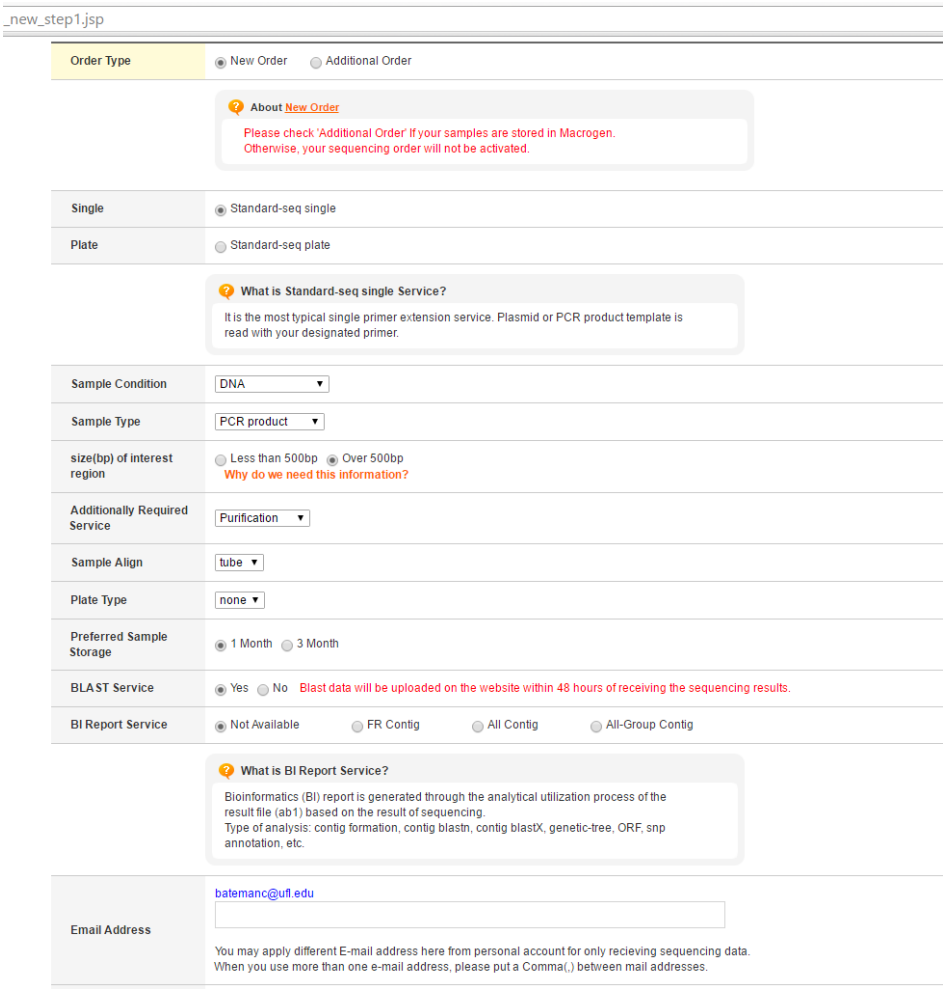
Sanger Sequencing

Obtaining a sequence from a third-party company

Sequencing is rarely performed by the lab performing the PCR. Many large universities have their own sequencing cores which handle sequencing for all researchers across the campus, or from private research institutions. There are also international companies which perform sequencing- and typically very quickly (2 days), cheaply, and accurately. Examples of these companies are Macrogen (South Korea) and GENEWIZ (US).

We have elected to use Macrogen for sanger sequencing.

After creating your account, fill in the correct information as below for standard Sanger sequencing.



Once you have completed the submission forms, arrange for shipment of the PCR product using DHL or TNT. This section needs to be revised after submissions have been completed successfully.

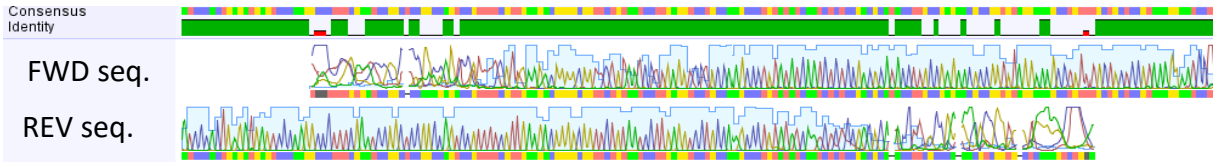
Phylogenetic analysis for identification

Introduction

Phylogenetic analysis is a complex topic, which is covered by entire books. The following is an extremely brief and largely inadequate overview of the typical process.

Assembling forward and reverse sequence contigs

During PCR, the length of an amplicon sequence will not exceed a certain length- usually 1000 base pairs as a maximum. In order to maximize the length of a sequence obtained, a site/locus in the DNA is copied from two directions- the primers copy DNA towards each other, so that the resulting sequences will overlap and increase the total length of the sequence obtained. When two sequences overlap in this way, they are called "contigs."



Notice that at the end of each sequence, the computer becomes unsure about what base pair was observed by the machine. These regions must be checked visually to see whether the colored peaks begin to overlap. These ends of the sequence should be trimmed as they are not reliable sources of information. If there are multiple overlapping peaks throughout a sequence read, your sample may have been contaminated with other DNA.

Once contigs are assembled and a reliable consensus sequence, it can be used in BLAST.

Identifying closest sequence match using BLAST

Copy and paste your consensus sequence into the BLAST by visiting blast.ncbi.nlm.nih.gov/Blast.cgi then click "nucleotide blast," and past your sequence into the box. BLAST is a tool, not a method (it stands for Basic Local Analog Search Tool).

BLAST will locate the closest sequence match in the Genbank database, but it does not identify organisms. Note there are two important columns in the results that help you decipher your matches: "Identity" and "Query Coverage." Identity shows how much of your sequence matches a sequence in the database, and Query Coverage indicates how much the two sequences overlap.

blast.ncbi.nlm.nih.gov/Blast.cgi

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Hypothenemus hampei haplotype SM1 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	739	739	100%	0.0	100%	GU133363.1
Hypothenemus hampei haplotype AM-10N cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	734	734	100%	0.0	99%	GU133361.1
Hypothenemus hampei haplotype CR1-COL1 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	728	728	100%	0.0	99%	GU133362.1
Hypothenemus hampei haplotype GUA-3N cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	728	728	100%	0.0	99%	GU133360.1
Hypothenemus hampei haplotype NGO-2N cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	728	728	100%	0.0	99%	GU133343.1
Hypothenemus hampei haplotype Bra-2N cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	723	723	100%	0.0	99%	GU133358.1
Hypothenemus hampei haplotype Bra30 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	717	717	100%	0.0	99%	GU133359.1
Hypothenemus hampei haplotype KAW8 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	717	717	100%	0.0	99%	GU133344.1

To actually identify an organism, a phylogenetic analysis must be performed.

Aligning sequences from closely-related species

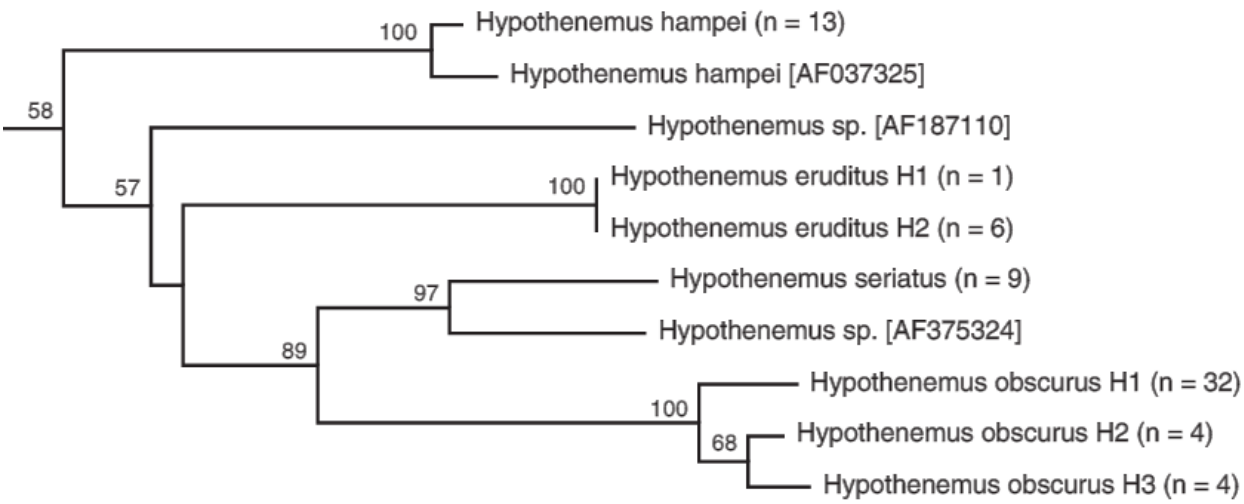
Before performing a phylogenetic analysis, you must find the most closely-related species to your organism in question. Only closely-related species with sequences of the same DNA site/locus available in a sequence database (such as Genbank) can be used.

Search the Genbank database for the most closely related species based on your initial BLAST results, and copy their DNA sequences of the same locus you sequenced, into your sequence analysis program. For example, if your BLAST results returned *Hypothenemus hampei* using the COI locus, search for COI sequences from other *Hypothenemus* species, including additional *H. hampei* COI sequences if multiple are available.

Once you have many sequences from the same locus of closely related species, the sequences must be aligned using a sequence analysis program. When the alignment is finished, all sequences must be overlapping. If there are parts of any sequences which does not align over all other sequences in the alignment (even if the sequences are different in the parts where they overlap), the ends must be trimmed so all parts of all sequences overlap.

Performing a phylogenetic analysis

A phylogenetic analysis is a way of inferring how organisms evolved over time. A phylogenetic tree is a visualization of this inference, as in the figure below. These analyses are outside the scope of this guide, but there is ample information online on this topic.



Sequence and Phylogenetic analysis programs

There are free tools available online for phylogenetics, and for-purchase programs which are typically easier to use and contain many more features (most of them unnecessary).

Online analysis: http://www.phylogeny.fr/

Mesquite: http://mesquiteproject.org/

MEGA: http://www.megasoftware.net/

Geneious (for-purchase): http://www.geneious.com/

Ordering equipment

Most equipment in the lab already has catalog numbers on them. These are good references for re-ordering equipment and consumables.

General, all

1. Thermo scientific pipettes . Nos: 14 488 013, 14 488 016, 14-488-019
2. Pipette stands (2) No.:F161401
3. Pipette tips ..... No.: 05-408-186, 05-408-190, 05-408-196
4. Lab gloves ..... No.: 19-130-1597C (A-E sizes)
5. BrandTech Scientific PCR tubes No.:781332
6. Microcentrifuge tubes.....No.: 022363204
7. Hyclone PCR-quality water ..... No.: SH30538.01
8. Fisher desktop microcentrifuge ..... No.: 75002436
9. Mini vortex..... No.: 02-215-365
10. Fisher Scientific Isotemp Freezer ..... No.: 13-986-148

DNA extraction

11. Extraction solution ..... No.: E7526-24ML
12. BSA.....No.:SH30574.01
13. Life Technologies TAE Buffer ..... No.: B49

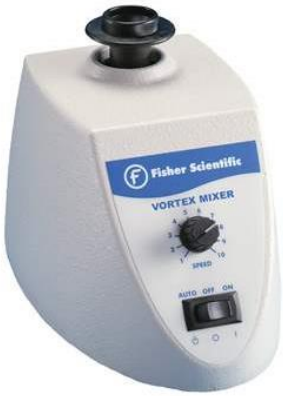
PCR and cleanup

14. DNA polymerase (Taq) and components .....No.: 50-443-954
15. Exosap-IT PCR Cleanup .....No.: 78201 1 ML'
16. Eppendorf Mastercycler Pro..... No.: E950030010

Gel electrophoresis

17. Agarose, analytical grade .....No.: BP2410-100
18. SYBR Green dye for DNA staining.....No.:50513
19. Perfectsize 5 Prime 100bp DNA ladder No.:2500340
20. Electrophoresis gel rig .....No.: S98617
21. Hoefer electrophoresis power supply ..... No.: PS300BNA
22. Clarit-E with UV Transilluminator, 254/365nm.....No. EL4050
23. SYBR green transilluminator filter ..... No.: EL4090

Photo glossary:

Microentrifuge tube (1.5mL)	
PCR tubes (.2 mL)	
Desktop microcentrifuge	
Vortex	

Electrophoresis gel rig