Nemours | Alfred I. duPont Hospital for Children
Nemours Children's Clinic

BCL

Biomolecular Core Facility
AI Dupont Hospital for Children, Rockland Center One, Room 214
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Pyrosequencing Submission Guidelines

Currently used by investigators for SNP/mutation detection analysis, allelic frequency, haplotyping and short-read sequence analysis.

Sample Submission

Contact the Core (651-6712**) at least a day in advance** to sign up for the Pyrosequencer. Please bring your sample submission sheet with you the day of your run.

Primer design

The Pyrosequencing Assay Design version 1.0 software is available to Core users for designing PCR and sequencing primers. Other primer design programs such as Mac Vector or Oligo 6.0 can be used to facilitate the design of the PCR primers. The PCR product should be between 100-300bp (the smaller the better). The primers should be 18-25 bp in length with a GC content and melting temperature about the same as the GC content and melting temperature as the product. In addition, they should be more GC rich in the 5'-end than the 3'-end for good specificity. Be sure to check the primers for potential self-annealing or hairpin loops and dimers. One primer of the pair is 5' biotinylated. The choice of which primer to biotinylate will depend on the direction of the sequencing primer, that is the biotinylated primer will be in the opposite direction of the sequencing primer. Check the biotinylated PCR primer extra carefully for hairpin loops and duplexes, as excess will cause increase background in the Pyrosequencing assay. The sequencing primer is designed for you via the Pyrosequencing Web site. It is highly recommended that investigators attend a Training/Software session in order to familiarize you with the Pyrosequencing Web site. The sequencing primer is located a few base pairs away from the SNP of interest. It is possible to sequence more than one SNP with each sequencing primer. In addition, multiplexing several PCR primers together in a single tube is also possible. Please contact the Core director, Dr. Katia Sol-Church to set up a consultation before setting up a multiplex reaction.

PCR conditions

It will be necessary to optimize your PCR reaction. A good starting place is 10ng DNA, 125uM each dNTP in a 50ul reaction. Concentration of primers should be 0.2uM (i.e. 10pmol in a 50ul reaction). Pyrosequencing recommends the use of TaqGold polymerase. Vary the annealing temperature (54°, 57° and 60° are a good starting point) and magnesium concentration (1.5, 2.0 and 2.5mM are good starting points) so that you produce a clear strong PCR band without excess primers, primer-dimers or other non-specific products. If your desired fragment is very GC rich, the addition of 5% DMSO or the incorporation of deoxyinosine (dI) for deoxyguanosine (dG) in a 3:1 (dI:dG) ratio may help your PCR reaction. In addition, GC rich templates often need a higher annealing temperature and lower magnesium concentration to amplify well. Annealing

temperature of 57 ° and magnesium concentration of 2.0mM are good starting points for GC rich templates.

You will need to set up 3 additional controls for each assay. One control is the template minus the sequencing primer. The other 2 contain water in place of template plus or minus primers.

It is possible to multiplex several PCR primers together in a single tube. Multiplexing is more complex and involves the use of additional controls. Please contact the Core director, Dr. Katia Sol-Church to set up a consultation before setting up a multiplex reaction.

TaqGold is gradually activated during the PCR reaction and requires more cycles than other Taq polymerases. In addition it is necessary to completely consume all of the biotinylated PCR primer before the Pyrosequencing assay. Forty five (45) to 50 cycles are recommended for best yield.

Typical PCR reaction (with 2.0mM MgCl final concentration)

10xPCR bufferII	5ul
MgCl (25mM)	4ul
dNTP (2.5mM)	2.5ul
DMSO	as needed
Primer A (10uM)	1ul (10pmol)
Primer B (10uM)	1ul (10pmol)
TaqGold	0.3ul
dH20	
DNA sample	10ng
Reaction total	50ul

Thermocycler Program: (approx. 1hr 45 min)

95°	5min
Cycle: 45x	
95°	15sec
Ta	30sec (annealing temperature)
72°	15sec
72°	5min
4°	hold

Following the manufacture guidelines, the Researcher will set up and perform the initial PCR reaction and the pyrosequencing reaction. The Core will provide the vacuum tool, which must be used in Room 214 Rockland Center One. Please sign up for the machine and coordinate the use of the vacuum tool and lab bench space **at least one day in advance**.

Results

Each plate takes 11 min. to run. A computer is available in the Core for the analysis, exporting and printing of your data.

Consultation
Consultation with the Core director, Dr. Katia Sol-Church is encouraged before starting Pryosequencing.

Fee
The cost per plate is $30.00. This charge includes a fee-for-use of the instrument and replacement filters for the vacuum tool.
For high volume discount, please contact the Director, Dr. Katia Sol-Church.
Consultations are free of charge for AIDHC investigators and affiliates.
Non AIDHC affiliated customers will be charged a 10% surcharge.

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