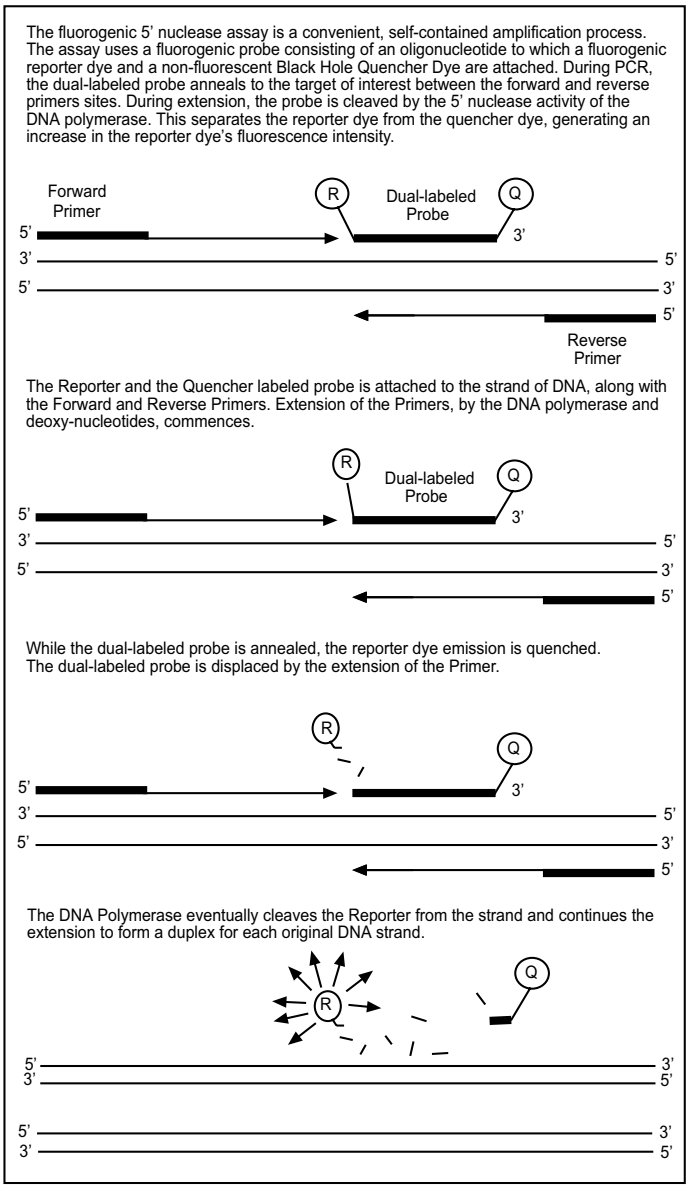
Fluorescence Monitoring of PCR

5' Nuclease Assay & Probe/Primer Design



Here is the basic scheme of the assay. The first step of each PCR cycle is the dissociation of the two strands of the DNA from each other, also termed "melting". Using this as a reference, the reasons for the guidelines will be apparent.

It becomes clear looking at this diagram, the importance of having a probe with a melting temperature (T_m) greater than that of the primer pair by 8-10°C, and having the T_ms of the primers very similar to each other. The goal is to allow the probe to anneal to all the targets, saturate the sample, without the primers annealing at the same time, so this step is performed at a temperature too high for the primers to anneal, but low enough that the probe will. When the temperature is at the anneal temperature, below the T_m of the primers, the primers will extend and cleave off the probe. You don't want the probe coming off simply because of temperature. You want the probe to come off because it was annealed to target and forced off.

The size of the amplicon is defined by the distance between the 5' ends of the two primers. When selecting the amplicon, it is better if it is ~100bp, because they amplify more efficiently than longer ones and this one aspect is the most important in the efficiency of the entire experiment. This is for several reasons. First of all, if the amplicon is short then the PCR materials will not be taxed too early in the PCR run. Under these conditions, there will be plenty of materials for many cycles of PCR forming an exponential growth curve; threshold will be clean, because competition for materials in the reactions will not control the curve. The 3' end of the primer that is on the same strand as the probe should be, optimally, 5 bases from the 5' end of the probe, but within 10 bases is acceptable; if the amplicon is short, this requirement is much easier to meet.

For all oligonucleotide sequences:
Runs of four or more nucleotides are not recommended, especially G. The interaction between the nucleotides can cause the oligonucleotide to fold in on itself producing secondary structure, which will be detected as multiple peaks on a PDA. During HPLC purification, which is based on the trace from the

PDA, oligonucleotides may be flushed as an unsuccessful synthesis based on multiple peaks. In general, if purification and analyses are difficult, based on secondary structure, then the ability of the sequence to find and anneal to target is compromised.

The sequences are not longer than 30 nucleotides so to calculate the T_m, use the "Nearest-Neighbor" method.

Probe Design Guidelines:
It is important that there is not a 5'G. It has been shown that guanosine quenches the adjacent fluorophore.

It is recommended to pick the probe that has more Cs than Gs, due to the strong interaction that G displays with itself.

Primer Design Guidelines:
It is important that the primers specifically anneal. The last 5 nucleotides on the 3' end of the primers could anneal non-specifically if their collective binding power is strong enough. This is why more than 2 Gs, 2 Cs or 1 GC is not recommended; Although, there are finer points to this rule. The following is the base stacking energy of the sequence of the 3' end of the primer:

GCGCG=CGCGC>GGGGG=CCCCC>GCGAT>GGGAT>>GAGAG etc.
So, if you have three G's or C's that are not all together it is okay.[1]

It is important that the primers do not form duplexes with each other or with the probe. Pay close attention to the Gs and Cs.

Avoid a 3' thymidine, since it is more prone to mis-priming than the other nucleotides

The forward and reverse primers should be as close to the probes as possible without overlap with the probe

Designing a Set[2]

Starting with the sequence you want to study, there are several steps that must be taken to ensure that the portion that is targeted is specific. You ought to run your sequence through a program to remove repetitive DNA before you make primers (with any primer program). The sequence should be in FASTA form. For multiple sequences, FASTA looks like this:

>Sequence1
ACGTGCGCGATCGCCTGCTAGGCGTACGTCGCAG
GCGATCGATGTGCTAGATCAGATGACA
>Sequence2
GGGCTAGATTAGCACCACATACATCGCTCA

RepeatMasker is available free to academic users and for a fee to others at:
http://ftp.genome.washington.edu/cgi-bin/RepeatMasker

[1] A. Yeung, Fox Chase Cancer Center
[2] M. Roy, Raven Biosciences

For tandem repeats:
http://c3.biomath.mssm.edu/trf.html

Primer3

There are various Probe/Primer set design software packages available. One such program, Primer3, is extremely reliable, fast, easy to use, and free. Primer3 is from the Whitehead Institute at MIT. It is available online for free. The software is available for download. You can run it in a Unix environment for large quantities of probe/primer sets. The following directions are for the design of a set optimized for an amplicon of length ~100bp. If you need clarification, within the program, each section is linked to definitions simply by double-clicking on them. Here is the link to the current version of Primer3:

http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi

The first cell is a drop-down menu, where you can specify the origin of the sequence you are studying:
RODENT
HUMAN
NONE

The large box is for the sequence. This can be copied and pasted out of GenBank, where it is in **FASTA** form.

There are three boxes that are below this. Check them all for a probe/primer set. If you want to design a primer set for Sybr Green, gel-based PCR, or you want to design the primers first and then design the probe, then just check the two primer boxes and don't check the "hyb oligo" box.

Excluded Regions: This is to exclude portions of the sequence. This tag is useful for tasks such as excluding regions of low sequence quality or for excluding regions containing repetitive elements such as ALUs or LINES (long interspersed elements) or SINES (short interspersed elements).

Product Size: 50 min 60 opt 90-100 max

Number to Return: default is 5, increase this, to get more choices.

General Primer Picking Conditions

Primer Tm: 58 min 60 opt 60 max
Product Tm: use default
Salt Concentration: 60.0 (this will account, somewhat, for the 5mM [Mg++], which strongly affects the annealing of the oligo and therefore the Tm)
Poly-X: Set to 3 (this eliminates runs of a single nucleotide of 4 or more)

Hyb Oligo (Internal Oligo) Per-Sequence Inputs

Hyb Oligo Excluded region: don't bother, you have insured a small amplicon and the probe is between the primers. Also any excluded regions have been entered above.

Hyb Oligo (Internal Oligo) General Conditions
Hyb Oligo Size: 20 min 24 opt 30 max
Hyb Oligo Tm: 68 min 70 opt 70 max (8-10°C higher than primer Tm entered above)

Salt Concentration: 60.0 (this will account, somewhat, for the 5mM [Mg++], which strongly affects the annealing of the oligo and therefore the Tm)
Poly-X: Set to 3 (this eliminates runs of a single nucleotide of 4 or more)
Hyb Oligo Mis Hyb Library: another drop-down menu, as at the top, pick origin:
RODENT
HUMAN
NONE

Hit "**Pick Primers**" button. Allow the program to work, your possible sets will be returned, shortly.

Sets with a 5' G
You might find that several sets have a probe with a 5'G. You have several choices at this point:
-You can take off the G if the next nucleotide in is not a G, but compare the Tm of the original against the one without the G, to make sure it is still 8-10°C above the primers.
-You can copy and paste the primers of the set you like, back into Primer3, at the top where you checked all three boxes, originally. Simply have the program pick your probe for these primers.
-You can choose to not use the set

Check the T_m s of the oligos with a separate program. The most efficient method is "Nearest-Neighbor" which is what Primer3 utilizes. The GC% calculators are ineffective for oligonucleotides under 30 nucleotides such as a TaqMan probe because GC interactions are important in folding but the interactions between all bases that "neighbor" each other are not taken into account. There is one problem, however, Primer3 uses [Mg++] = 1.5 mM, whereas the average concentration is 5 mM. This concentration has a huge effect on the T_m. There is a "Salt concentration" box. Testing primer set designs for accuracy has shown that making the "Salt concentration" value 60 mM can help correct the T_m s. There is only one T_m Calculator that has separate entries for oligo, Na+, and Mg++ concentrations and it has been tested by comparing actual melts against the theoretical values. This calculator can be found at the following link:

http://www.rnature.com/oligonucleotide.html

After designing oligos, do a BLAST search or a similar analysis to determine the specificity. Both right and left need to match to get a positive result:

http://www.ncbi.nlm.nih.gov/blast/Blast.cgi

Choose amplicon with minimal secondary structure. This is important, since secondary structures could affect the efficiency of the reaction. In any real-time application it is desirable to obtain a 100% efficiency of the amplification reaction, which means that each time a cycle is completed exactly twice as many amplicons should be present in the reaction. If the secondary structure is thermodynamically more stable than the oligo target hybridization, hybridization of the target will be disfavored. Secondary structure could also prevent polymerase read through. Keep the GC content between 20 and 80%. G-C-rich sequences are susceptible to non-specific interactions that may reduce reaction efficiency and could produce non-specific signal in SG assays. A recommended program to test for secondary structures is Mfold. This is a versatile folding program for use in analyzing amplicons, linear probes over 30 nucleotides, and Molecular Beacons. It is found at the following link:

http://www.bioinfo.math.rpi.edu/~mfold/dna/form1.cgi.

How to use Mfold:

1. name the sequence
2. enter sequence into large box
3. In "folding temperature" enter the annealing temperature of the reaction (~60°C).
4. In "Ionic conditions" change the units to "mM" and enter the [Na+} as 50 mM and [Mg++] for 3-6 mM.
5. enter email (the program sends you nothing, but this must be entered)
6. Click the "fold DNA" button (next to happy face)
7. You will get a list of structures. A well-designed sequence will yield only one form. The "PNG" form is a nice form in which to view the structure and the melting temperature of the structure will appear in a separate window.

To judge what structures are strongly favored, look at the delta G value: +10 strongly not favored, -10 strongly favored, with approximately linear variation of those extremes in between. If an amplicon secondary structure is unavoidable the primer annealing temperature should be increased.

Once the probes and primers are designed, as outlined above, the cycling conditions are always identical. After the initial denature time (depending on the *Taq*-Polymerase) the assay needs to be run for 15 to 20 sec at 95°C and for 60 sec at 60°C. For some assays an extension of 45 sec seems to be enough. Data have to be acquired at annealing temperature.

For an assay with a dual labeled probe, optimal performance is achieved by selecting the primer and probe concentration that provide the lowest Ct-value and the highest increase of fluorescence compared to the background.

Concentration Optimization[3]

The most important thing, which has to be optimized, is the primer/probe concentration and its ratio.
The primer concentrations should be optimized by spanning an initial concentration range of 50nM – 900nM. Because the individual efficiency of the forward and reverse primer can vary, their respective concentrations must vary to compensate. Therefore all permutations of a selected number of primer concentrations must be tested. For instance, there are nine possibilities of how forward and reverse primer concentrations could be combined for the following individual concentrations: 50nM, 300nM, and 900nM:
F/R: 50/50, 50/300, 50/900, 300/50, 300/300, 300/900, 900/50, 900/300, 900/900.

The probe concentrations should be optimized by spanning an initial concentration range of 50nM – 250nM.
Three probe concentrations (50nM, 100nM, 250nM) should be combined with the nine forward and reverse primer concentrations above yielding 27 reactions. These should be run with the above described cycling parameters. You will have your initial set of raw data so quantify each reaction. After quantitation analysis, the curve with the lowest Ct value and the highest amplification should be chosen for further experiments.

[3] T. Kaiser, Corbett Research

Probe and primer optimization for the dual labeled probes

1. Make up a Master Mix containing everything except for the probe and primers.

Master Mix

	Stock Concentration	Final Concentration	Per reaction	Master Mix (x34)
Water			6.75	229.5
10X buffer	10X	1X	2.5	85
$MgCl_2$	50 mM	3 mM	1.5	51
dNTPs	2.5 mM	0.2 mM	2	68
Forward Primer	*	*	5	
Reverse Primer	*	*	5	
Probe	#	#	1	
Taq-Polymerase	5u/µl	0.05U/µl	0.25	8.5
DNA			1	34
Total			**25**	

* Primer stock concentrations are 4.5, 1.5 and 0.25µM
  Primer final concentrations are 50, 300 and 900nM (See Pt 4 below)

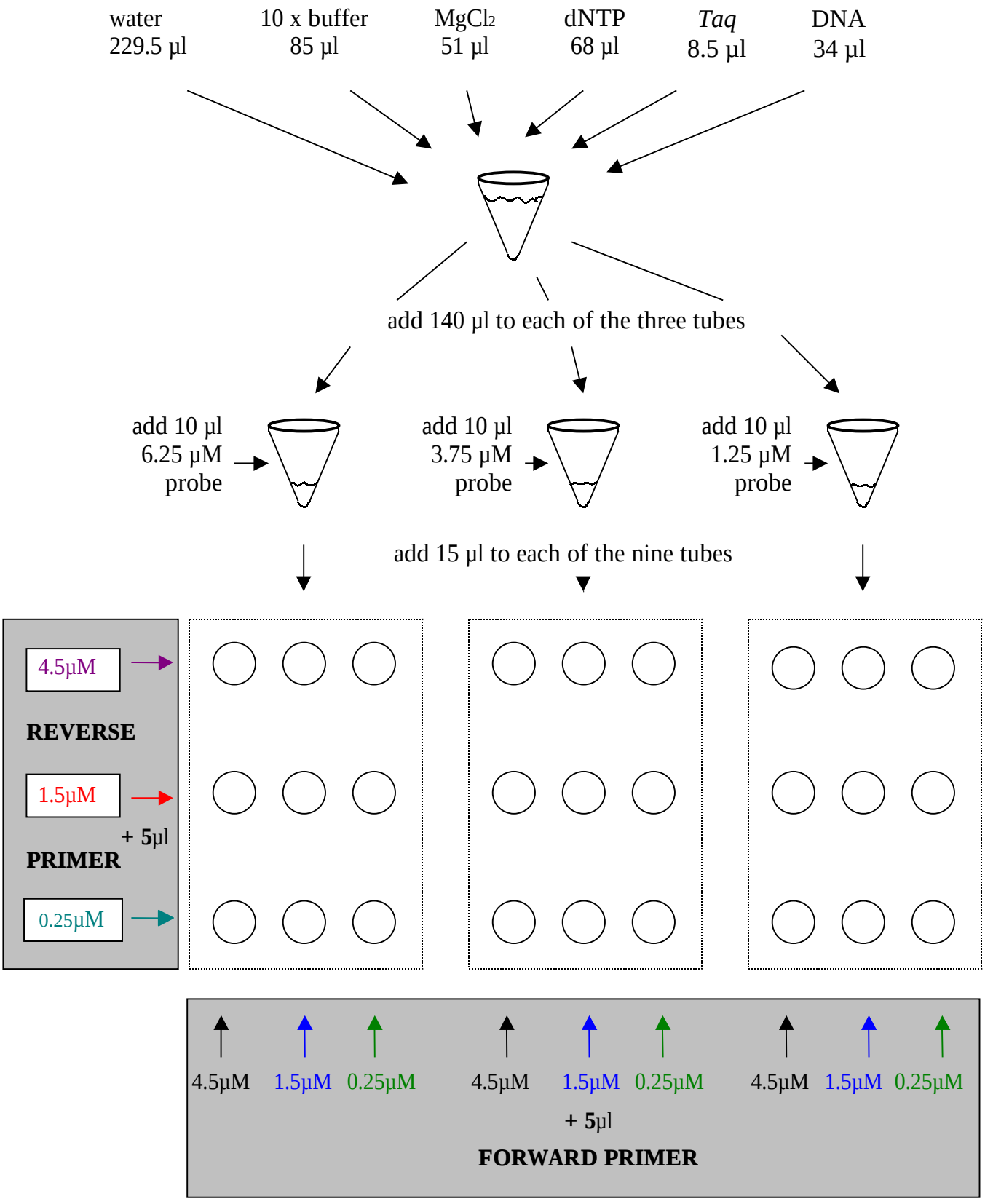
Probe stock concentrations are 6.25, 3.75 and 1.25 µM
  Probe final concentrations are 250, 150 and 50nM (See Pt. 2 below)

2. Divide the Master Mix into 3 x 140µl and add the 10µl of probe at concentrations of 6.25, 3.75 or 1.25µM (Final 250, 150 and 50nM, respectively).

3. Aliquot 15µl of the three Master Mixes into reaction tubes.

4. To the reaction tubes add 5ul of each primer to give final reaction concentrations outlined in the table below. The primer stock concentrations are 4.5, 1.5 or 0.25µM and give final concentrations of 50, 300 or 900nM respectively.

	Forward primer		
Reverse Primer	900	300	50
900	900/900	300/900	50/900
300	900/300	300/300	50/300
50	900/50	300/50	50/50


water
229.5 µl
10 x buffer
85 µl
MgCl2
51 µl
dNTP
68 µl
Taq
8.5 µl
DNA
34 µl
add 140 µl to each of the three tubes
add 10 µl
6.25 µM
probe
add 10 µl
3.75 µM
probe
add 10 µl
1.25 µM
probe
add 15 µl to each of the nine tubes
4.5µM
REVERSE
1.5µM
+ 5µl
PRIMER
0.25µM
4.5µM
1.5µM
0.25µM
4.5µM
1.5µM
0.25µM
4.5µM
1.5µM
0.25µM
+ 5µl
FORWARD PRIMER