PCR

Primer

The most important thing to establish a new PCR is to choose your primers. There are programs in the net like Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) that do this for you however, primers should be between 15 and 25 bp long with an optimum at 20 bp. They should anneal netween 55 and 65°C with an optimum at 60°C, and they should have an GC percentage of about 50%.
Primer are delivered lyophilized and with tare solubilized in Sigma water as 100µM Stock. Aliquot the primers from the stock as to reduce repeated thawing of the stock. The primers are used at a 25mM concentration in the mix, so always freshly dilute the primers.

PCR Setup

All ingredients are to be equilibrated to 37°C, vortexed, spun down and directly put on ice. The entire PCR is pipetted on ice!!!!! Especially the Taq polymerase has to be kept ice cold at all times and therefore is pipetted last!!!

Ansatz:

SAMPLE NUMBER COMPONENT	1X ANSATZ [ML]
Leber cDNA	
10 x Buffer	2,5
50 mM $MgCl_2$	0,75
5 mM dNTPs	1
Primer F [25µM]	1
Primer R [25µM]	1
DMSO	
Taq Pol	0,2
H_2o up to 25µl	17,55

PCR condition depend on size of the fragment, primers and DNA used.

Taq polymerase needs 30'' to synthesize 500bp. So set your elongation time appropriately.
The best annealing temperature for the primers has to be tested over a temperature gradient.
For genomic DNA always use 35 cycles, for Plasmid DNA 15 cycles are sufficient.

PCR condition - example:

Denature		94°C 2'
Denature	35x	94°C 30"
Anneal		65°C 30"
Elongate		72°C 90"
Elongate		72°C 5'

PCR conditions can vary, if the initial temperature gradient does not work you can additionaly titrate various compounds: 1-5µl DNA; 1- 2.5mM $MgCl_2$, 0-10% DMSO. Have a look at Henry Suns paper (*Tung-Tien Sun*, Excessive trust in authorities and its influence on

Box 1 | The 'N+(N–1) rule': optimizing a reaction condition by titration

Titration of key elements			
Enzyme A	5 µg	1, 2, 5*, 10 µg	$N_1 = 4$
$MgCl_2$	2 mM	1, 2*, 5 mM	$N_2 = 3$
KCl	25 mM	10, 25*, 50, 100 mM	$N_3 = 4$
pH	8	7, 8*, 9	$N_4 = 3$

Conventional rule
$4 \times 3 \times 4 \times 3 = 144$

N + (N-1) rule
$4 + (3–1) + (4–1) + (3–1) = 11$

By definition, titration means to determine the optimal concentration of a component in a reaction mixture by measuring the effects of systematically changing its concentration. In the example given in the main text, several of the reaction components are particularly important — that is, 5 µg of Enzyme A, 2 mM $MgCl_2$, 25 mM KCl and pH 8.0 (whatever the buffer). We can therefore carry out the titration by comparing the effects of varying: the enzyme concentration at 1, 2, 5* and 10 µg ($N_1 = 4$; meaning that for variable number one — that is, the enzyme — we will test four concentrations); the $MgCl_2$ concentration at 1, 2* and 5 mM ($N_2 = 3$); KCl at 10, 25*, 50 and 100 mM ($N_3 = 4$); and pH at 7, 8* and 9 ($N_4 = 3$) (see figure; asterisks highlight the originally recommended amount). To do all these titrations properly — that is, for us to change only one variable at a time (a cardinal rule) — this would require a total of $N_1 \times N_2 \times N_3 \times N_4$ (or $4 \times 3 \times 4 \times 3$) = 144 reactions. This is, of course, impractical. Fortunately, as we know that all the data points in a biochemical reaction almost always form rather smooth curves, instead of jumping all over the place (barring poor pipetting skills), we do not need to carry out all of these 144 reactions. Rather, we can take a shortcut using a procedure that we call the 'N+(N–1) rule', which tells us the minimal number of reactions that we need to do in a titration. In the above example, we can add up the four Ns, subtracting one from each of them except the first one: $[(N_1) + (N_2–1) + (N_3–1) + (N_4–1)]$. So, we need a total of 4 + (3–1) + (4–1) + (3–1) = 11 reactions only (see BOX 2 for how these 11 reactions can be designed).

Box 2 | The 'N+(N–1) rule': constructing a table to titrate key components

To titrate the components listed in BOX 1, you can make a table with 11 rows for a total of 11 reactions (as calculated in BOX 1; see table). Above these rows enter: Enzyme A (with 4 columns indicating the test concentrations — that is, 1, 2, 5* and 10 µg); $MgCl_2$ (1, 2* and 5 mM); KCl (10, 25*, 50 and 100 mM); and pH (7, 8* and 9) (see BOX 1; asterisks highlight the originally recommended amount). To titrate the enzyme, select 1 µg for tube 1, 2 µg for tube 2, 5 µg for tube 3, and 10 µg for tube 4; for all four tubes, we will select (or gamble by using) the recommended 'standard' values for all other reagents. The results from these four tubes will titrate the enzyme concentration by changing only a single variable (the enzyme concentration) at any one time. Note that tube 3 (highlighted by‡) is unique, in that all four components happen to be of the standard value (this is the only reaction that would have been done if we had decided not to titrate). The result of this tube can therefore be used, repeatedly, when we titrate all other components (therefore 'N–1'). Next, we can titrate $MgCl_2$, by selecting 1 mM (tube 5), skipping the recommended 2* mM (this tube would be identical to tube 3), and selecting 5 mM (tube 6). As noted before, for these two tubes we will keep all the other variables constant by selecting the recommended values. Do the same for titrating KCl (tubes 7–9) and pH (tubes 10 and 11). You can then carry out the experiment, and enter the results (in this case, the incorporation of a radioactive precursor in counts per minute (cpm)) in the last column (see BOX 3 for how to plot the results).

Reaction	Enzyme A (µg)				$MgCl_2$ (mM)			KCl (mM)				pH			Results (cpm)
	1	2	5*	10	1	2*	5	10	25*	50	100	7	8*	9	
1	+					+			+				+		30
2		+				+			+				+		60
3‡			+			+			+				+		65
4				+		+			+				+		68
5			+		+				+				+		50
6			+				+		+				+		60
7			+			+		+					+		75
8			+			+				+			+		50
9			+			+					+		+		40
10			+			+			+			+			62
11			+			+			+					+	55