

July 31, 1989

I am sending a dried sample (a few micrograms) of the plasmid pAKtaq. It was constructed by amplification of the Taq DNA polymerase coding region plus a bit of 3' noncoding region using the oligos contained on the attached sheet. These oligos contained extrinsic restriction site sequences at either end (EcoRI upstream, BglII downstream) to facilitate cloning into the EcoRI/BamHI site of expression plasmid pTTQ18 (Amersham, map enclosed). We have been using it in a DH1 strain of E. coli, but I suspect you can use it in any lab strain.

In practical terms the clone is quite easy to use. Simply grow cultures to about $A_{600} = 0.2$ (doesn't seem critical) and add IPTG to 0.5 mM and grow overnight. We use LB+amp and it is possible to get away with less IPTG if desired. Final yield of enzyme by the procedure outlined below is about 200,000 to 300,000 units per liter of culture when compared to the Cetus enzyme. Take care to always titrate the enzyme, since my early preps have not worked at too high an enzyme concentration. Whether this is an oddity of the polymerase or a contaminant, I don't know, but it is stable at 94° and seems to copurify with the activity. All numbers given below are per liter of cell culture.

1. Harvest, resuspend in 200 ml buffer A (50 mM TRIS, pH 7.9, 50 mM dextrase, 1 mM EDTA), and repellet cells.
2. Resuspend cells in buffer A containing 4 mg per ml lysozyme - 40 ml. Let stand at room temperature 10-15 min.
3. Thoroughly mix in 40 ml buffer B (10 mM TRIS, pH 7.9, 50 mM KCl, 1 mM EDTA, 1 mM PMSF freshly diluted from 144 mM stock in DMF, 0.5% Tween 20, 0.5% NP40). incubate at 75° for 30-60 min, depending on how long it takes the extract to come to temperature.
4. Spin in cold GSA rotor (Sorvall) 15 min, 8000 rpm. Save supernatant.
5. Polyethyleneimine (PEI) precipitation. Each extract behaves a little differently with PEI, so it is necessary to titrate the amount of PEI each time. PEI is kept as a 10% (w/v) stock at 4° after pHing to 7.5 with HCl and filtering if necessary. To 200ul aliquots, add 0.5, 1, 2, 4, 8 or 16 ul of 10% PEI and vortex. Let stand on ice 10 min and spin in cold microfuge 10 min. Remove supernatant and resuspend pellets as well as possible in buffer C (20 mM HEPES, pH 7.9, 1 mM EDTA, 0.5 mM PMSF, 0.25% Tween 20, 0.25% NP40) containing 0.25 M KCl. Let stand 10 min and respin. Do your favorite rapid assay on dilutions. (See attached sheet for mine.) I find about a final of 0.15% PEI brings most of the enzyme down. Please note that the titration is essential because too much PEI blocks the precipitation.
6. Add the appropriate amount of PEI dropwise to the rest of the prep while stirring and let stand on ice 30 min. All subsequent step are performed in the cold room. Spin at 8000 rpm for 20 min in GSA rotor.
7. Resuspend the pellet in 20 ml buffer C plus 0.025 M KCl using a Dounce homogenizer with a loose pestle. This pellet is rather tough, but perservere. Respin. Resuspend pellet in 0.15 M KCl in buffer C the same way, then give a couple strokes with a tight-fitting pestle. Let stand 20 min and respin. The supernatant will contain most of the activity, although you can squeeze a little more out of the pellet by eluting with 0.25 M KCl (and it will be dirtier.)

8. Dilute the supernatant with buffer C to 50 mM KCl and load on a column of BIOREX 70 equilibrated with the same buffer. 10-15 ml packed resin per original liter of cell culture seems about right. Wash the column with buffer C/50 mM KCl and elute the Taq DNA polymerase with buffer C containing 200 mM KCl. The protein in this fraction should be more than 90% Taq DNA polymerase and the pool should be 20-100 units per microliter.

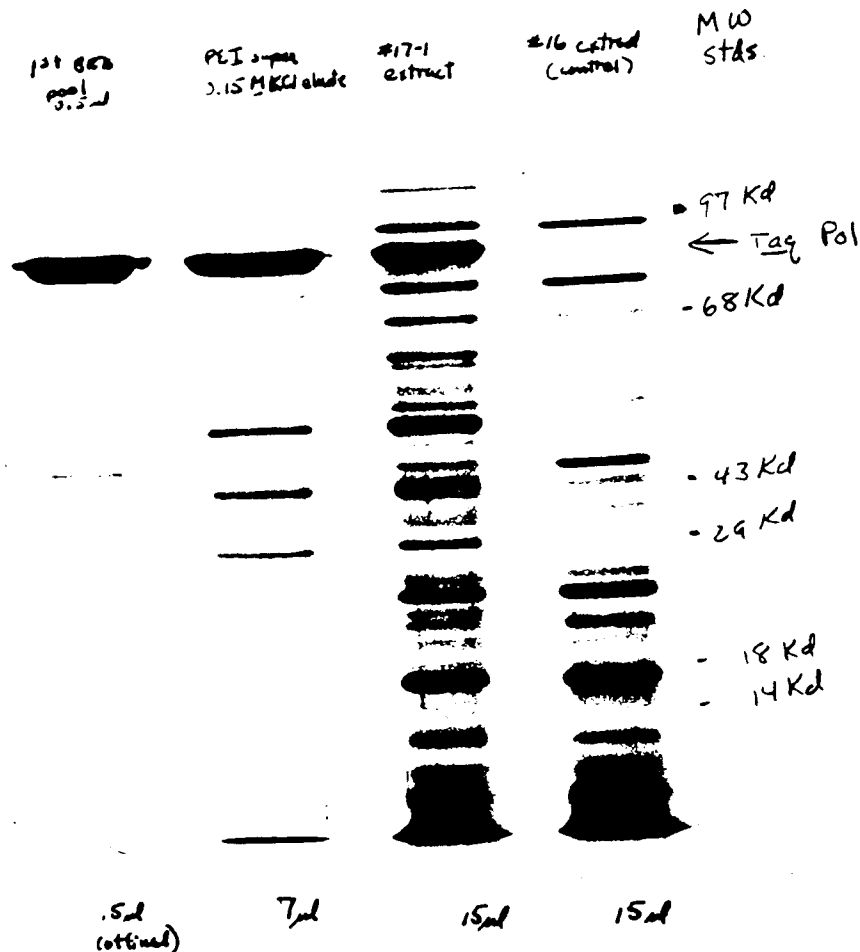
50% glycerol
20 mM HEPES (pH 7.5)
0.1 mM EDTA
0.5 mM PMSF
1 mM DTT
100 mM KCl

9. Dialyze the pooled enzyme into storage buffer and aliquot. This can be stored at either -20° or -80° after quick-freezing. Activity is stable to at least 5 rounds of freeze/thaw. I use 1-2 units for PCR, about 0.1 unit for sequencing M13 and about 10-50 units for yeast genomic DNA sequencing, but titrations are best. I only see the odd high concentration inhibitory effect with PCR assays, where a little too much produces a high molecular weight smear and way too much produces intensely staining small molecular weight material.

Shown below is a Coomassie-stained protein gel of various fractions from a prep. If you want to test your clones for correctness, it is probably easiest to make mini-induced extracts and assay them.

Good luck.

P.S. - Several groups contributed to making this clone: Mary Ellen Bruck in David Ginsberg's lab; Alexandra Krikos in Myron Levine's lab; and my self.



Rapid assays of Taq DNA polymerase

5 ul reactions: 0.5 ul 10X Taq pol. buffer (ala Cetus)
0.5 ul dNTP mix (0.1 mM dATP, 1 mM each dGTP, dCTP, dTTP)
0.5 ul (1 ug) any ss M13 viral DNA
0.1 ul (0.1 ug) M13 forward primer
0.1 ul (1 uCi) alpha-³²P-ATP
2.3 ul H₂O
4.0 ul cocktail (made up in advance)
+1.0 ul Taq DNA pol. dilution
37^o, 1 min; 65-70^o, 3 min; add 10 ul deionized formamide + dye(s)
100^o, 3 min; load on mini-Sanger gel and run XC about 2 inches in.
Expose wrapped gel for 1-2 hours with intensifying screen.

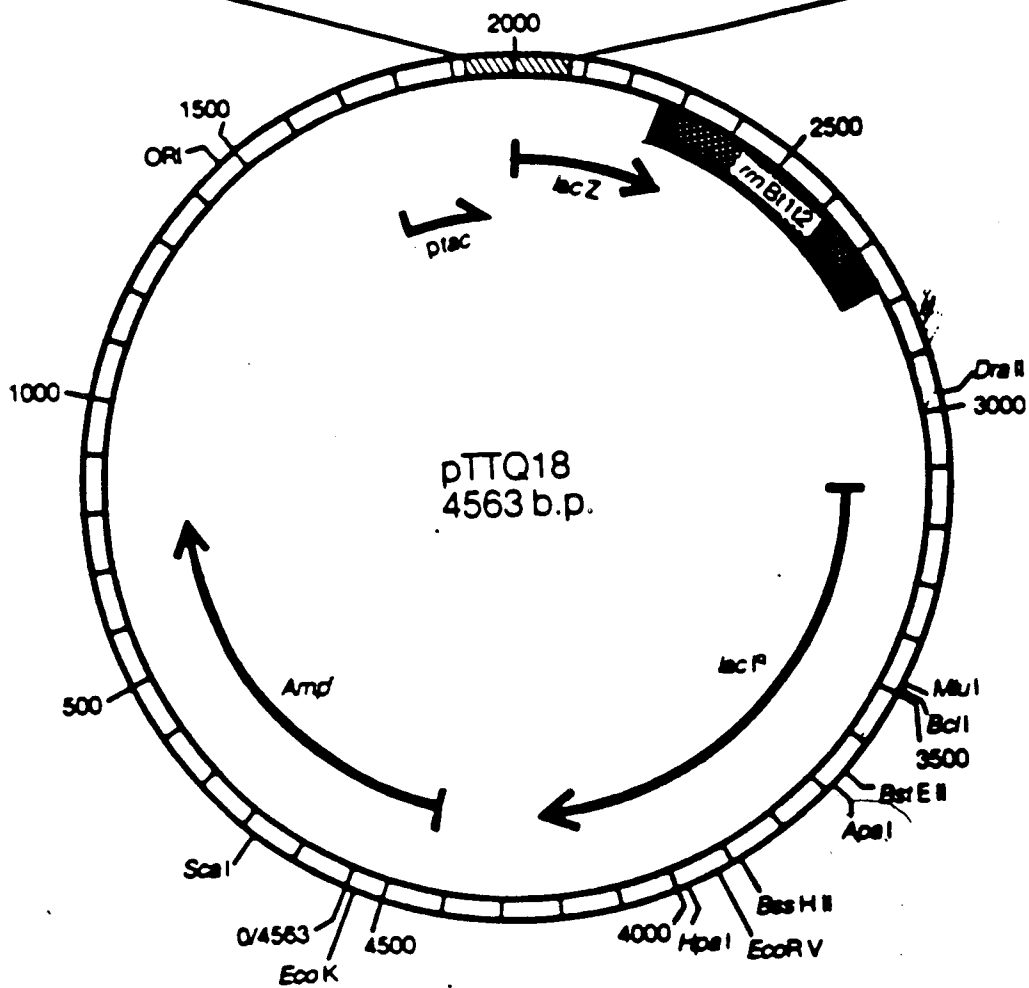
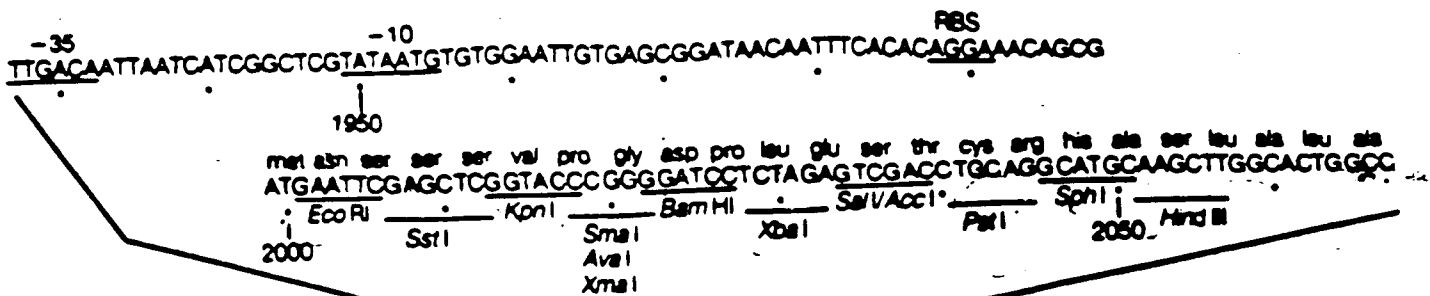
Primers used for Taq DNA polymerase gene amplification.

The sequence of the gene was taken from Lawyer et al. (1989) J. Biol. Chem. 264, 6427-6437. The extraction procedure was adapted from the same source. Underlined sequences correspond to the extrinsic EcoRI and BglII restriction sites introduced for cloning.

Upstream: 5' - CACGAATTTCGGGGATGCTGCOOOCCTCTTTGAGOOCAAG

Downstream: 5' - GTGAGATCTATCACTOCTTGGOGGAGAGOCAGTC

RESTRICTION MAP OF PLASMID pTTQ18



mB112: *E. coli* mB operon transcription terminators

ptac: Synthetic tac promoter
 lacP: Lac repressor gene
 lacZ: Beta-galactosidase alpha fragment gene

The map shows the positions of restriction enzyme sites that occur only once in pTTQ18.

Sites not present: AarII, AflII, AseIII, AvrII, BclI, BglII, BsmI, ClaI, DraIII, EcoA, EcoD, Eco47, EspI, MluI, NaeI, NcoI, NdeI, NheI, NotI, NruI, PstII, SmaI, SfiI, SnaI, SnaBI, SpeI, SstII, StuI, StyI, Tth111I, XhoI, XmaI.