

**PCR-based gene disruption in *Saccharomyces cerevisiae*
using SFH- or LFH-PCR and *kanMX* selectable marker**

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Application protocol

Use of the dominant resistance marker *kanMX* in *Saccharomyces cerevisiae* transformations

1. INTRODUCTION

PCR-generated DNA molecules consisting of a marker cassette with short flanking homology regions to the target locus (SFH-PCR) are now used in several laboratories for directed gene alterations in *S. cerevisiae*. This technique has been termed PCR-targeting and was firstly described for PCR-generated DNA molecules with the *S. cerevisiae* *HIS3* marker flanked by 35-50 bp of DNA homologous to the target locus (Baudin et al., 1993). Transforming DNA is generated in a PCR reaction using marker DNA as template and two 60-70 b oligonucleotides as primers. The oligonucleotide primers used have the following features (see Fig.1): From 5' to 3', primer 1 (primer 2) has 35-45 b homologous to the 5'-site (3'-site) of the *Saccharomyces cerevisiae* genomic target locus followed by 18-19 b of sequence derived from the 5'-region (3'-region) of the marker. The resulting PCR-products thus contain the marker module flanked by 35-45 bp of DNA homologous to the genomic target locus.

The use of the homologous *HIS3* marker in PCR targeting experiments, however, resulted in a large number of false positives (His^+ transformants) that are due to the integration of the marker at its natural chromosomal site (most *his3* alleles carry point mutations or small deletions and not a complete deletion of the locus including promotor and terminator). This problem became solved when *kanMX*, a completely heterologous dominant resistance marker, was used as selector module (Wach et al., 1994). These *kanMX* modules are hybrids of the coding sequence of the *kan^r* gene of transposon *Tn903* coding for aminoglycoside phosphotransferase (Oka et al., 1981) and transcriptional and translational control sequences from the *TEF* gene of the filamentous fungus *Ashbya gossypii* (Steiner, 1991; Steiner and Philippsen, 1994). Aminoglycoside phosphotransferase activity renders *S. cerevisiae* resistant to the drug geneticin (G418) (Jimenez and Davies, 1980). *KanMX* is one component in a new series of reporter/marker plasmids (pFA, plasmids for Functional Analysis) that can be used as templates for SFH-PCR. Due to the heterology of the *kanMX* selector module in pFA, correct disruptions of *S. cerevisiae* genes with PCR-generated *kanMX* molecules flanked by 35 bp of *Saccharomyces cerevisiae* homologous DNA at either side, occur with a very low level of false integrations (Wach et al., 1994). In addition, this marker cassette can be used independently of auxotrophic mutations in the transformed *S. cerevisiae* strain. Further studies on fidelity of PCR targeting with *kanMX* have shown that less than 2% of the obtained transformants were G418-resistant due to mis-integration of the *kanMX* module or spontaneous resistance (A. Brachat, A. Wach and P. Philippsen, unpublished data).

Systematic studies on the length of the flanking regions added to the marker have shown that 30 bp of homology at each side are sufficient for successful targeting (Manivasakam et al., 1995). A modification of this technique by using direct repeat-containing oligonucleotides for PCR-amplification of the heterologous *Kluyveromyces lactis* *URA3* marker cassette has been reported and it was shown that such a construct can be excised from the genome by mitotic recombination (Laengle-Ronault and Jacobs, 1995).

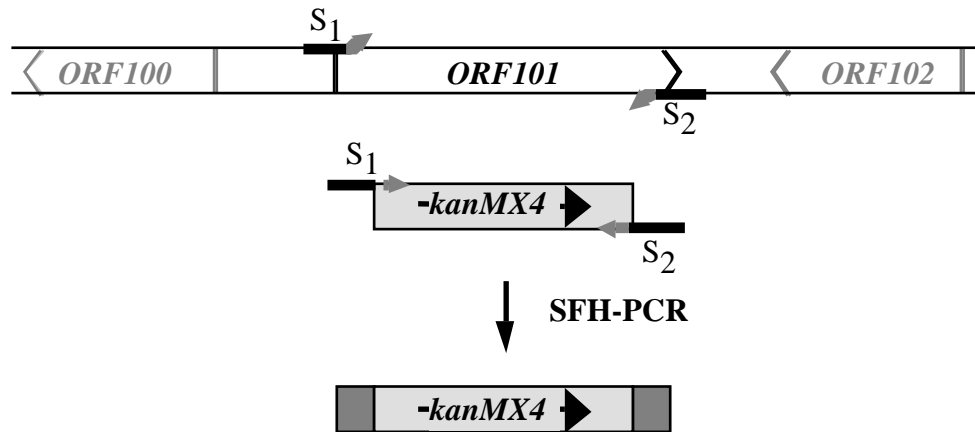


Fig.1 - SFH-PCR to produce marker DNA flanked by small homology regions.

Note: To be more flexible in marker choice, we derive these sequences from the 5'- (3'-) side of the multiple cloning site in pFA vectors rather than of the marker itself. The sequence homologous to pFA in primer 1 is: 5'-CGTACGCTGCAGGTCGAC-3' (SunI, PstI, Sall restriction sites) and that in primer 2: 5'-ATCGATGAATTCGAGCTCG-3' (ClaI, EcoRI, SacI restriction sites). Restriction enzyme sites may also be included at the 5'-extremities of the primers used to amplify the marker module. This facilitates subsequent PCR-product cloning. However, non-homologous DNA sequences at the ends of the transforming DNA with small flanking homology regions usually decrease the integration frequency of these molecules into the *Saccharomyces cerevisiae* genome.

Successful targeting of SFH-PCR products depends on perfect homology the short ends (30-40 bp) of the transforming DNA and the target locus. Thus, sequence polymorphism in different strains will restrain homologous recombination when PCR-made molecules with small homology regions are used. Therefore, as an alternative to SFH-PCR, a new PCR strategy was developed in our laboratory in Basel (Wach, 1995), and also independently by others (Amberg et al., 1995). The long flanking homology technique we have introduced (LFH-PCR), uses dual-step PCR to produce sufficient quantities of a gene targeting cassette with much longer flanking homology than those obtained with the more straightforward single-step SFH-PCR. The principle of LFH-PCR is shown in Fig.2.

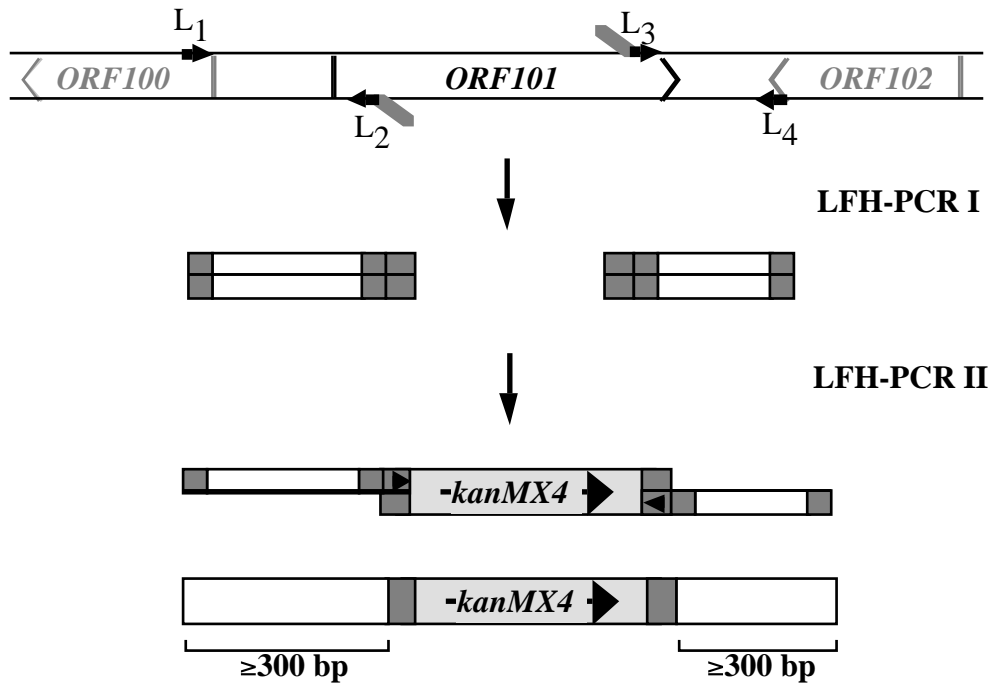


Fig.2 - LFH-PCR to produce marker DNA flanked by long homology regions with primers L1 and L4 (outer primers) and L2 and L3 (inner primers).

Note: We recommend the following primer characteristics when using this technique:

primer L1 : 20-mer of individual choice (300-800 b upstream of ATG)

primer L2: 5'-GGGGATCCGTCGACCTGCAGCGTACCATNNNNNNNNNNNNNNNNNNNN-3'

< derived from pFA MCS ><* 20-25 b >
from target ORF immediately upstream
of second codon (*ATG was included)

primer L3: 5'-AACGAGCTCGAATTCATCGATGATATGANNNNNNNNNNNNNNNNNNNN-3'

< derived from pFA MCS ><* 20-25 b >
from target ORF immediately downstream
of last codon (*stop was included)

primer L4 : 20-mer of individual choice (300-800 b downstream of stop codon)

Gene targeting cassettes with long flanking homology regions 5' and 3' to the marker are synthesized in two separate reactions. In the first PCR, two pairs of primers and *Saccharomyces cerevisiae* genomic DNA (or a cosmid clone) are combined in the same reaction tube. One primer pair (L1 and L2) is used to amplify the 5'-region directly upstream of the target gene's second codon (5'-PCR fragment) and the other primer pair (L4 and L3) to amplify the DNA region immediately downstream of its last codon (3'-PCR fragment). The sequences of the 3'-halves (19-22 bases) of the *inner* primers L2 and L3 are derived from the *Saccharomyces cerevisiae* target locus. However, these primers also carry 5'-extensions (20-25 bases) derived from the marker module (open bars in the primer symbols in Fig.2) in order to generate in the 5'- and 3'-PCR fragments short overlapping homologies to the selection marker. In the second PCR with marker DNA as template, one strand of the 5'-PCR fragment and one strand of the 3'-PCR fragment then serve as *long* primers to produce the ORF targeting cassette. In this complex reaction, several PCR fragments are produced: *i.e.* marker with 5'- and 3'-extension (LFH-PCR fragment) but also marker modules with only 5'- or 3'-extensions are synthesized (side products). The amount of the LFH-PCR fragment can be boosted by addition of an

excess of the two *outer* primers (L1 and L4, indicated in the top of Fig.2). The yield of the ORF targeting cassette is not as high as with SFH-PCR. However, the longer homology regions lead to improved transformation efficiencies (e.g. in *Saccharomyces* strains with low levels of sequence heterogeneity). Moreover, construction of plasmids carrying the ORF deletion cassette and the cognate ORF, as requested in the EUROFAN program, can be immediately started with the LFH-PCR fragment as discussed below.

G418-resistant transformants made with either SFH- or LFH-PCR fragments are then checked for the correct integration of the marker at the target locus. This can be done either by Southern blot analysis or much faster, and as reliably, by PCR. Before the transformants can be analyzed, G418-resistant colonies must be streaked out and regrown on G418-containing plates until single colonies are visible (clonal purification). This also ensures the elimination of false positives (background growth).

This analytical PCR-method is based on a proposed technique for rapid analysis of the mating type of *S. cerevisiae* cells (Huxley et al., 1990). Purification of the chromosomal DNA is not necessary. Instead, whole *Saccharomyces cerevisiae* cells are used. The principle of this method is shown in Fig.3.

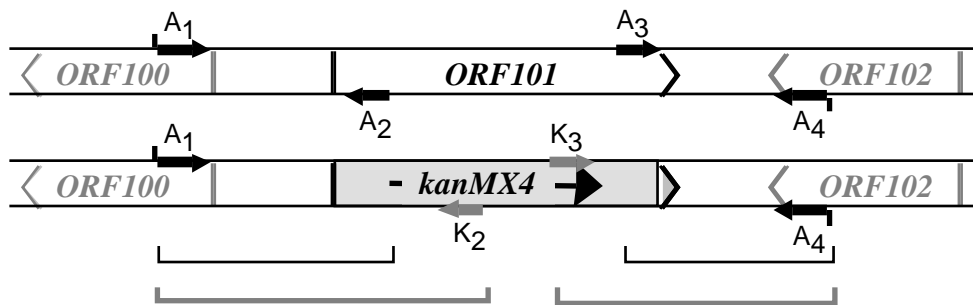


Fig. 3 - PCR-Analysis with diploid *Saccharomyces cerevisiae* cells to identify clones with correctly targeted marker DNA

Since the DNA sequence at the target locus, as well as that of the marker gene are known, it is possible to design oligonucleotides that bind outside of the target locus (A1 and A4), within the target locus (A2 and A3) or within the marker module (K2 and K3). In diploid *Saccharomyces cerevisiae* transformants with correctly integrated marker, one of the two copies of the target locus has been replaced by the marker module. In a PCR with *Saccharomyces cerevisiae* cells (genomic DNA) as template and using three of these oligonucleotides as primers (either A1, A2, and K2 or K3, A3, and A4), the correct integration of the marker DNA is visualized by the appearance of two PCR products of predictable length: one characteristic for the wild type allele (A1-A2 or A3-A4 amplification product) and a second fragment characteristic for the mutated allele (A1-K2 or K3-A4 amplification product). Incorrect transformants will only yield the amplification products of the wild type allele. For complete verification of the integration both novel joints must be tested. The data obtained with such analytical PCR experiment are probably more reliable than data obtained by Southern blot analysis.

2. EXPERIMENTAL PROCEDURES

A. Transformation of *S. cerevisiae* with PCR products.

I. PCR-Synthesis of disruption cassettes with small flanking homology (SFH-PCR):

1. Set up 100 µl PCR-reaction mix [10 mM TRIS-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 0.2 mM dNTP, 1 µM Primers, 0.2-0.5 µg (1 nM) plasmid template (*e.g.* pFA6-kanMX4)] and vortex. Divide the mix into two 50 µl aliquots and run PCR.
2. Heat PCR-Mix to 94 °C for 2 min and then add, 1 µl DNA polymerase-Mix (see below). We use *Taq DNA polymerase* and also add 0.5 U of DNA polymerase with 3'-5' exonuclease activity, like *Vent DNA polymerase*, to decrease the error rate during DNA polymerization and to prevent non-specific 3' nucleotide overhangs (this mix is prepared prior to the PCR by mixing 25 U *Taq DNA polymerase* with 5 U of *Vent DNA polymerase*).
3. PCR conditions are:

120 sec, 94 °C (initial denaturation)	
(hot start)	
30 sec, 94 °C (denaturation)	<-- this sequence
30 sec, 54 °C (annealing)	<-- is repeated
90 sec, 72 °C (elongation)	<-- 20 times
120 sec, 72 °C (final elongation)	
samples are then cooled to 2 °C	
4. Add 5 µl of 3M Na-Acetate followed by 120 µl of ethanol, vortex and centrifuge at 12000 rpm for 15 min.
Optional: Samples can be treated with phenol/chloroform prior to precipitation.
5. Wash pellet with 70% ethanol, air-dry for 10 min, and resuspend pellet in 10 µl of TE.
6. Analyze 1 µl by agarose gel electrophoresis.
7. Use 5 µl (usually 1-5 µg of DNA) to transform *Saccharomyces cerevisiae* (see below).

II. PCR-Synthesis of disruption cassettes with long flanking homology (LFH-PCR):

1. Digest 1 µg pFA6-kanMX4 with *NotI*.
2. First PCR (production of long 5'- and 3'-homology regions):
Set up 50 µl PCR-reaction mix [10 mM TRIS-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 0.2 mM dNTP, 1 µM Primers (four primers, L1, L2 and L4 and L3, are used in the same reaction), 0.1-0.5 µg template (*e.g.* plasmid clone, cosmid clone or *Saccharomyces cerevisiae* genomic DNA)], vortex, and run PCR.
3. Heat PCR-Mix to 94 °C for 2 min and then add, 1 µl DNA polymerase-Mix.
3. PCR conditions are:

120 sec, 94 °C (initial denaturation)	
(hot start)	
15 sec, 94 °C (denaturation)	<-- this sequence
15 sec, 54 °C (annealing)	<-- is repeated
30 sec, 72 °C (elongation)	<-- 20 times
samples are then cooled to 2 °C	
4. Analyze the resulting PCR products by agarose gel electrophoresis.

Note: It is not necessary to further purify the PCR products from the first LFH-PCR. They can be immediately used in the second LFH-PCR. However, when the two products differ in quantity or when the amount of remaining primer in one or the other reaction is too high, purification of the PCR products is required.

5. Second PCR (linking of 5'- and 3'-homology regions from first PCR to marker DNA):
Set up 50 µl PCR-reaction mix [10 mM TRIS-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 0.2 mM dNTP, 1 µM Primers (L1 and L4), 1 µl of first PCR (this is usually equivalent to 0.1-0.2 µg of each PCR-fragment), 0.1-0.5 µg template (*e.g.* pFA6a-kanMX4, *Not* I-digested)], vortex, and run PCR.
6. Proceed as described under 2.
7. PCR conditions are:

120 sec, 94 °C (initial denaturation)	
(hot start)	
30 sec, 94 °C (denaturation)	<-- this sequence
30 sec, 54 °C (annealing)	<-- is repeated
120 sec, 72 °C (elongation)	<-- 25 times
240 sec, 72 °C (final elongation)	
samples are then cooled to 2 °C	
8. Precipitate and analyze the DNA as described above.

III. *Saccharomyces cerevisiae* transformation

III.1 Preparation of competent cells

We usually prepare competent *Saccharomyces cerevisiae* cells as described (Gietz and Woods, 1994)

1. Inoculate a 5 ml YPD preculture of *Saccharomyces cerevisiae* from a single colony and grow overnight at 30 °C.
2. Determine the cell density of the preculture by counting the cells.
3. Inoculate a 50 ml YPD culture with 1 10⁴ cells/ml and grow cells at 30 °C for 10 generations (about 16 hours).
4. Count the cell density and collect cells by centrifugation at 1500 g for 5 min in 50 ml Falcon tubes.
5. Resuspend the pellet in 20 ml of sterile water, and centrifuge again at 1500 g for 5 min.
During this period, prepare 1 ml of 100 mM LiAc from 1 M LiAc stock solution and 2 ml of 100 mM LiAc/40% PEG3350 from 1 M LiAc and 50% (w/v) PEG3350.
6. Resuspend the pellet in 1 ml of sterile water and transfer the suspension into an Eppendorff-tube.
7. Centrifuge Eppendorff-tube at 1500 g for 5 min, remove the supernatant, and resuspend the cells to a density of 2 10⁹ cells/ml in 100 mM LiAc (this solution is made up freshly from a 1M stock solution). Do this by suspending the cells in 200 µl of 100 mM LiAc, determine the resulting total volume with a Gilson pipette, and adjust to the final volume.
8. Incubate the cells at 30 °C for 20 min.

III.2 Transformation

1. Denature sheared herring sperm carrier DNA by boiling for 10 minutes. Place immediately on ice after boiling.
2. To each of the PCR-DNA containing Eppendorff-tubes, add 5 µl of carrier DNA (10 µg/µl).

3. Add 50 µl LiAc-treated competent *Saccharomyces cerevisiae* cells to each tube, vortex and incubate 20 min at 30 °C.
4. Pipette 300 µl PEG/LiAc solution into each tube, vortex and incubate for 20 min at 30 °C.
5. Heat shock the transformation tubes at 42 °C for 20 min (the duration must be optimized for every strain). Note: The addition of DMSO to 10% (v/v) final concentration prior to the heat shock improves the efficiency of transformation by up to 10-times in some strains.
During the heat shock period, fill one culture tube per transformation with 3 ml of YPD.
6. Centrifuge the transformation tubes at 5000 rpm for 1 min in a microfuge, remove the PEG/LiAc supernatant with a Gilson pipette, and resuspend the cells in 1 ml YPD.

III.3 Selection

The procedure for selection of G418-resistant clones that we originally described (Wach et al., 1994) was worked out for only one *Saccharomyces cerevisiae* strain. The selection procedure has to be optimized when you work with different strains. If *Saccharomyces cerevisiae* strains like FY1679, W303 or CEN.PK2 are used we apply the following procedure:

1. Resuspend the cell pellet in 1 ml YPD, transfer this suspension into the prepared YPD culture tubes and incubate cultures for 2-3 hours at 30°C with shaking (i.e. for one doubling of cell number). Note: Do not waste empty Eppendorff-Tubes. They will be needed after pre-incubation for the collection of cells.
2. Take 1.5 ml aliquots of each culture and transfer them into Eppendorff-Tubes, centrifuge at 5000 rpm for 1 min in a microfuge, remove 1.1 ml of the supernatant and resuspend the cells in the remaining volume of supernatant.
3. Plate the suspension on YPD plates containing 200 mg/l G418.
4. Incubate plates at 30 °C for 2-3 days. After this period, a few big (3-4 mm in diameter) as well as many small (0.2-2 mm in diameter) colonies are seen on the plates. The small ones are the background of abortive transformants that arise, most probably, from cells that carry non-integrated *kanMX* DNA and produced sufficient aminoglycoside-phosphotransferase to inactivate G418 during a few rounds of division.
5. Purify transformed cells from background by streaking out cells from each big colony on YPD-G418. Only those clones that can grow out to **colonies from single cells** are putative positive integrants. Note: Do not transfer the colonies into patches on the new plate.

B. PCR-analysis of G418-resistant transformants

I. Analytical PCR:

1. Transfer single colonies from the restreaked putative transformants into PCR reaction tubes. Do this by picking cell material corresponding to 1 mm in diameter of a single colony with a sterile yellow pipette tip and patch the cells on the wall at the bottom of the tube. Include also one sample with wild type cells as control.
2. Cell pellets are then heated in a microwave for 1 min.
Alternatively, resuspend cells in 50 µl of Zymolyase (20 U/ml) and incubate for 10 min at room temperature. Collect the cells by centrifugation at 5000 rpm for 1 min in a microfuge, remove the supernatant, and heat the cells for 5 min at 92 °C. Note: This method is a little bit more time-consuming but leads to higher amounts of PCR product as compared to the "microwave" procedure.

3. Add 25 μ l PCR-reaction mix [10 mM TRIS-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 0.2 mM dNTP, 1 μ M Primers A1,A2, K2 or A3, K3, A4, 1-2 U Taq DNA Polymerase] to each pellet, vortex, and run PCR. Note: The reaction suspension must be kept on ice until the PCR is started since proteases secreted by the *Saccharomyces cerevisiae* cells might inactivate the DNA polymerase).
7. Start PCR
PCR conditions are:

120 sec, 94 °C (initial denaturation)	
30 sec, 94 °C (denaturation)	<-- this sequence
30 sec, 50 °C (annealing)	<-- is repeated
90 sec, 72 °C (elongation)	<-- 30 times

samples are then cooled to 2 °C
8. The samples are then centrifuged at 10000 rpm for 1 min in a microfuge, the mineral oil is carefully removed with a pipette, and 3 μ l of gel loading buffer (50 mM EDTA, 30% (v/v) glycerol, 0.25% (w/v) bromophenol blue) are added to the samples.
9. Vortex samples briefly and centrifuge again at 10000 rpm for 1 min to sediment the cells.
10. 10 μ l of each supernatant are then analyzed on an agarose gel.

Literature:

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Materials and Reagents:

YPD	yeast extract, Bacto	1	%
	peptone, Bacto	2	%
	glucose	2	%
YPD-G418 plates	YPD containing:		
	G418 (geneticin)	200	mg/l
	agar, Gibco	2	%
	(G418 was used from several suppliers - all work well)		
2x YT	yeast extract, Bacto		g/l
	tryptone, Bacto		g/l
	NaCl		g/l
2x YT-Amp plates and liquid	2x YT containing:		
	ampicilin	100	mg/l
	(agar, Gibco	2	% in solid media)
2x YT-Kan plates	2x YT containing:		
	kanamycin	50	mg/l
	agar, Gibco	2	%
NaAc	CH ₃ COO-Na	3	M
	CH ₃ COOH	2	M
	pH 5.4		
LiAc	CH ₃ COO-Li	1	M
PEG	PEG3350, Sigma	50	%
ssDNA	herring sperm DNA, 10 Boehringer Mannheim		mg/ml
Gel loading buffer	glycerol	30	%
	EDTA	50	mM
	bromphenol blue	0.1	%
Ethidiumbromide	10000x	10	g/l
dNTP	dATP, dCTP, dGTP, dTTP, New England Biolabs	2	mM each