Gene Cloning Using PCR

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2.1 Introduction

Since its invention, the polymerase chain reaction (PCR) has revolutionized the methodology for cloning genes. To clone a gene of interest, only sequence information of two distinct regions of the gene (or the protein) is needed to enable the design of primers for the amplification of the intervening sequence. Following amplification of a putative gene fragment, the respective PCR product can be used as a homologous probe for cloning the complete gene from a library. PCR has therefore rapidly replaced the previously used screening of libraries with oligonucleotide probes. Due to the fast progress in PCR technologies and applications, further developments today enable cloning of a complete gene including 5′- and 3′-untranslated regions by PCR alone. This is particularly important in organisms where the preparation of genomic DNA in quantities and qualities sufficient for the construction of a genomic library is difficult. In this chapter we will attempt to review the current state of gene cloning by PCR.

2.2 Tools Required

2.2.1 Available DNA polymerases

Several commercial suppliers offer a variety of thermostable DNA polymerases which differ in their proofreading activity, thermostability and terminal deoxynucleotide transferase (TdT) activity (i.e., DNA polymerases which add an additional non-templated nucleotide to the 3′-end of a DNA

 Applications of PCR in Mycology (eds P.D. Bridge, D.K. Arora, C.A. Reddy and R.P. Elander)

Table 2.1. Features of thermostable DNA polymerases.[a,b]

	Taq	Stoffel	r*Tth*	*Pfu*	Vent	*Pwo*
Optimal extension temperature (°C)	75–80	75–80	75–80	72–78	76	72
Extension rate at 72°C (kb min^{-1})	2–4	2–4	2–4	0.5–1	1	1–2
Reverse transcriptase activity	Minimal	Minimal	Mn^{2+} dependent	Minimal	Minimal	Minimal
Half-life (min) at 100°C	< 5				100	> 120
Half-life (min) at 97.5°C	10	20	2			
Half-life (min) at 95°C	40	80	20	d	400	
Mg^{2+}-ion PCR optimum (mM)	1–4	2–10	1.5–2.5	1.5–2.5	1–6	1.5–4
pH optimum for PCR (25°C)	9.0	9.0	9.0	> 8.0	8.8	8.8
dNTP PCR optimum (µM each)	40–200	40–200	40–200	100–250	200–400	200
KCl PCR optimum (mM)	50	10	75–100	10	10	25
Primer PCR optimum (µM)	0.1–1	0.1–1	0.1–1	0.1–0.5	0.4	0.3–0.6
Longest PCR product (kb)	> 10		> 10	25	15	7.5
dI-containing primers accepted	+			–	–	
5′–3′ exonuclease activity	+	–	+	–	–	–
3′–5′ exonuclease activity	–	–	–	+	+	+
Error rate (mutation frequency per base pair per cycle × 10^{-6})	8.0			1.3	2.7	e
Expandase activity[c]	+	+	+	–	–	–
Substrate analogues:						
dUTP	+	+	+	–		
deaza-dGTP	+	+	+	+		
biotin-11-dUTP	+		+	+		+
digoxigenin-dUTP	+		+			+
fluorescein-dUTP			+	+		+
ddNTP	+	+	+			
bromo-dUTP	+					
α-thionucleotides	+			+		

Continued on facing page

Table 2.1 continued

[a] *Taq* from *Thermus aquaticus*; Stoffel, a modified form of recombinant *Taq* DNA polymerase, with the N-terminal 289 amino acids containing the 5′→3′ exonuclease activity removed; *Tth*, from *Thermus thermophilus*; *Pfu*, from *Pyrococcus furiosus*; Vent from *Thermococcus littoralis*; *Pwo* from *Pyrococcus woesei*.

[b] This table was composed from a number of sources including publications, manuals, catalogues and data sheets (Lundberg *et al.*, 1991; Hu, 1993; Biotechnology catalogue, Perkin Elmer, New Jersey, USA; Promega catalogue, Madison, Wisconsin, USA; *Pfu* DNA Polymerase Instruction Manual, Stratagene, La Jolla, California, USA, and references therein; data sheets for Vent DNA polymerase from NEB, New England, USA; *Pwo* DNA polymerase from Boehringer Mannheim, Germany). The table is therefore incomplete.

[c] Expandase activity refers to the addition of a non-templated nucleotide to the 3′-end of blunt-ended DNA molecules.

[d] *Pfu* DNA polymerase remains > 95% active following a 1 h incubation at 95°C.

[e] Due to different assays to estimate the error rate no direct comparison is possible. The error rate of *Pwo* is similar to that of *Pfu* and Vent polymerases (Cha and Thilly, 1993).

strand). This enables the selection of the optimal polymerase for individual applications (Table 2.1). Polymerases with proofreading activity such as *Pfu* remove 3′-misincorporated nucleotides and so increase the accuracy of the reaction. Such polymerases may also increase the yield of amplicons as misincorporated nucleotides can inhibit the activity of the DNA polymerase (Barnes, 1994a). Conversely, proofreading activity can degrade primers (Lundberg *et al.*, 1991; Flaman *et al.*, 1994).

2.2.2 Source of DNA

An extensive search of literature from the past 5 years shows that most instances of the use of PCR in the cloning of fungal genes have used genomic DNA as a template. Since neither high quantities nor high quality of DNA are needed, a number of simple and rapid protocols can be used for DNA extraction. Virtually every type of fungal material can be used as a source of DNA, and procedures have been adopted for mycelia harvested from liquid cultures (Gruber *et al.*, 1990), solid media (Lecellier and Silar, 1994), fruiting bodies, herbarium materials (Bruns *et al.*, 1990) or from archaeological specimens (Rollo *et al.*, 1995).

2.2.3 Primer design

Design of primers is one of the key steps in the successful cloning of a gene. However, no absolute rules can be given that will guarantee the amplification of the desired fragment in sufficient quantities. The following features may be taken as guidelines for the design of primer pairs: (i) the length of primers should be between 20 and 30 bases; (ii) the GC content should be around 50%, and G and C nucleotides should preferably be randomly distributed within the primer. Over-long GC-stretches should be particularly avoided at

the 3′-end of the primer, as this can cause efficient but unspecific priming at any GC-rich region in the genome (Innis and Gelfand, 1990); (iii) both primers should have a similar GC content to minimize differences in annealing at a given temperature; (iv) stretches of poly(dR), poly(dY) and palindromic sequences should be avoided; (v) the sequences of the two primers must not be complementary; (vi) the concentration of both primers in the reaction should be between 0.1 and 0.5 μM. Differences in the concentration of the two primers should be avoided since this would favour the amplification of a linear single-stranded fragment.

Several computer programs are available which assist in the calculation of optimal annealing temperatures, GC-contents, secondary structures, primer-dimer formation and other properties of primers (e.g. GENE RUNNER 3.00, Hastings Software, Inc., 1994).

Even when the above guidelines are fully considered, primer design is, however, not straightforward. A given amino acid may be encoded by different triplets due to the degenerate nature of the genetic code (see Table 2.2). As a consequence, a mixture of primers ('degeneration') instead of a primer with a defined sequence is required. The degeneracy of the nucleotide triplet encoding a certain amino acid is an important issue in the design of primers: degenerate primers enable the amplification of related but distinct nucleic acid sequences as well as of targets for which only amino acid sequences are available (e.g. from protein sequence data or from multiple alignment of related sequences from different organisms). Obviously, the use of protein sequences containing amino acids with little degeneracy is desirable because it provides the greatest specificity. No general rules can be given for the degree of degeneracy that allows successful amplification. Even 1024-fold degenerated primers have been shown to work well in PCR (Knoth *et al.*, 1988).

Several approaches can be considered for increasing the specificity of the amplification using degenerate primers (Kwok *et al.*, 1994): (i) the primer pools may be synthesized as subsets where one contains either a G or C at a particular position, whereas the other contains either an A or T at the same position; (ii) the degeneracy of the mixed primer may be reduced by considering the codon bias for translation. For instance, codons having an A in the third position (e.g. CTA or TTA for leucine) are used only rarely (Table 2.2); (iii) degeneracy at the 3′-end of the primer should be avoided, because single base mismatches may obviate extension; (iv) the inclusion of deoxyinosine (I) at some ambigous positions may reduce the complexity of the primer pool. Several experiments have suggested that deoxyinosine might be an 'inert' base (Martin *et al.*, 1985) and its presence in an oligonucleotide sequence probably will not cause any disturbance in DNA duplex formation or result in destabilization of the duplex. However, pairing of dI with the four different bases is not equal: dI:dC >> dI:dA > dI:dT = dI:dG (Ohtsuka *et al.*, 1985). Furthermore, it has to be taken into account that deoxyinosine oligonucleotides cannot be used with some DNA polymerases, such as Vent or *Pfu* (Knittel and Picard, 1993).

Table 2.2. Codon usage in ascomycetous filamentous fungi.

Amino acid[a]	Codon	*A. nidulans*[b]		*A. niger*[b]		*N. crassa*[b]		*T. reesei*[b]	
		Number	%	Number	%	Number	%	Number	%
Gly	GGG	893	15	178	8	600	9	113	8
Gly	GGA	1323	22	386	16	910	13	183	14
Gly	GGT	1710	28	863	36	2267	34	240	18
Gly	GGC	2091	35	944	40	2981	44	801	60
Glu	GAG	3139	59	1019	71	4270	74	433	82
Glu	GAA	2221	41	414	29	1463	26	98	18
Asp	GAT	2348	47	691	41	2041	40	227	31
Asp	GAC	2642	53	991	59	3052	60	510	69
Val	GTG	1309	23	498	27	1083	18	205	23
Val	GTA	532	9	83	5	370	6	34	4
Val	GTT	1597	28	433	24	1474	25	161	18
Val	GTC	2167	39	810	44	3075	51	507	56
Ala	GCG	1519	20	357	15	1233	15	225	17
Ala	GCA	1430	19	266	11	763	10	151	12
Ala	GCT	2109	28	770	32	2061	26	286	22
Ala	GCC	2460	33	1022	42	3969	49	631	49
Arg	AGG	524	10	93	9	849	16	80	13
Arg	AGA	537	10	80	7	565	11	39	7
Ser	AGT	831	11	216	9	528	8	66	5
Ser	AGC	1380	19	531	22	1433	22	316	25
Lys	AAG	2930	69	1019	87	4283	84	528	92
Lys	AAA	1345	31	159	13	818	16	48	8
Asn	AAT	1296	37	282	22	849	23	151	19
Asn	AAC	2243	63	984	78	2906	77	632	81
Met	ATG	1905	100	493	100	2138	100	261	100
Ile	ATA	508	11	36	3	322	7	24	4
Ile	ATT	1656	37	385	29	1538	32	214	33
Ile	ATC	2354	52	906	68	2881	61	418	64
Thr	ACG	1097	20	316	16	1012	18	293	29
Thr	ACA	1189	22	190	9	724	13	102	10
Thr	ACT	1356	25	474	23	1055	19	200	20
Thr	ACC	1720	32	1054	52	2741	50	410	41
Trp	TGG	1150	100	412	100	1239	100	244	100
End	TGA	41	28	15	26	71	34	6	19
Cys	TGT	431	37	119	34	235	22	56	24
Cys	TGC	731	63	235	66	825	78	176	76

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Table 2.2 continued.

Amno acid[a]	Codon	*A. nidulans*[b]		*A. niger*[b]		*N. crassa*[b]		*T. reesei*[b]	
		Number	%	Number	%	Number	%	Number	%
End	TAG	53	36	16	28	34	16	14	44
End	TAA	52	36	26	46	103	50	12	37
Tyr	TAT	939	37	269	24	758	28	132	25
Tyr	TAC	1600	63	869	76	1939	72	400	75
Leu	TTG	1157	14	324	15	1191	15	96	10
Leu	TTA	496	6	26	1	250	3	5.0	1
Phe	TTT	1127	34	243	23	940	27	191	38
Phe	TTC	2148	66	834	77	2491	73	309	62
Ser	TCG	1247	17	346	15	1200	18	286	22
Ser	TCA	1038	14	119	5	515	8	103	8
Ser	TCT	1418	19	426	18	1036	16	200	16
Ser	TCC	1526	21	732	31	1885	29	318	25
Arg	CGG	887	17	148	14	585	11	71	12
Arg	CGA	897	17	84	8	428	8	92	16
Arg	CGT	1062	20	298	28	1026	20	102	17
Arg	CGC	1445	27	376	35	1807	34	209	35
Gln	CAG	2268	62	734	77	2553	71	524	81
Gln	CAA	1378	38	215	23	1056	29	124	19
His	CAT	975	45	190	32	678	31	58	17
His	CAC	1176	55	402	68	1497	69	275	83
Leu	CTG	1881	24	664	31	1448	18	365	36
Leu	CTA	769	10	103	5	409	5	24	2
Leu	CTT	1662	21	355	17	1509	19	140	14
Leu	CTC	2016	25	653	31	3094	39	379	38
Pro	CCG	1142	23	280	21	985	20	192	22
Pro	CCA	1103	22	116	9	687	14	102	12
Pro	CCT	1399	28	365	27	1169	23	216	25
Pro	CCC	1362	27	601	44	2167	43	346	40

[a]Three-letter code.
[b]Total number of genes used in the derivation of this data: *Aspergillus nidulans*, 146; *Aspergillus niger*, 57; *Neurospora crassa*, 208; *Trichoderma reesei*, 32.

2.2.4 Other reaction components

The PCR reaction requires magnesium-chelated dNTPs in a pH-stabilized environment. The concentration of each of the nucleotides should be in the range of 50–200 µM and should be balanced. Differences in concentration of the nucleotides may cause misincorporation and thereby decrease yield and

fidelity of the PCR (Ehlen and Dubeau, 1989). Most suppliers of thermostable DNA polymerases also supply the respective buffers, and hence their optimization is usually no problem. Changing the Mg^{2+}-concentration from 1 to 10 mM can have dramatic effects on the specificity and yield of an amplification. The presence of EDTA or other metal ion-chelating agents, which are used in some DNA extraction procedures (Möller *et al.*, 1992) and may therefore be carried over, lowers the effective concentration of Mg^{2+}. An excess of dNTPs has the same effect. The concentration of metal chelators and total dNTPs should therefore be taken into account when determining the final Mg^{2+} concentration required for PCR.

The use of 1–10% (w/v) dimethylsulphoxide (DMSO) in the PCR assay has been recommended (Scharf *et al.*, 1986; Chamberlain *et al.*, 1988), because it lowers the melting temperature of the dsDNA and hence facilitates strand separation. This may be particularly important in the denaturation of GC-rich DNA and help to overcome difficulties caused by DNA secondary structures (Hung *et al.*, 1990). However, the use of DMSO is not generally recommended, as it apparently increases the solubility of the mineral oil (frequently used as a protectant against evaporation) in the aqueous phase and thereby inhibits DNA polymerase (Linz, 1991). An alternative to the mineral oil layer is the use of a thermocycler with an integrated heated lid. Use of formamide (1.25–10%) can also facilitate primer–template annealing reactions and lower the denaturing temperature of DNA (Sarkar *et al.*, 1990).

Glycerol is also frequently included in the reaction system (5–20%, w/v), because it may improve the yield of the amplicon by stabilizing the DNA polymerase (*Instruction Manual for* Pfu *DNA Polymerase*; Stratagene, La Jolla, California). A similar stabilization can also be achieved by the addition of bovine serum albumin (10–100 μg/ml), which has the further advantage of binding fatty acids and phenolic compounds which may inhibit the PCR (Pääbo *et al.*, 1988).

2.2.5 Cycling parameters

In a typical PCR reaction, the double-stranded DNA is denatured by heating the sample to 94–98°C. The primers are allowed to anneal at the calculated annealing temperature (typically 40–60°C) followed by heating to the extension temperature (depending on the thermostable DNA polymerase used, 68–76°C). This cycle is repeated 25–40 times. Normally, these cycles are preceded by an initial denaturation step of 1–3 min and followed by a final extension step at the chosen extension temperature for 5–10 min. To avoid extension of unspecifically annealed primers at lower temperature while setting up the PCR mixture, a 'hot start' is recommended. This means that the enzyme is added to the reaction mixture after the initial denaturation step only. 'Hot start' has disadvantages, however, in that it is laborious when handling many samples

at the same time, and carries the risk of introducing contamination. As an alternative to 'hot start', *Taq* antibodies can be added to the reaction mixture. The antibody inhibits *Taq* polymerase during the setting up of the reaction mixture only because it is subsequently inactivated at the first denaturation step. Denaturation should be as short as possible to avoid inactivation of the thermostable DNA polymerase by prolonged exposure to elevated temperatures. On the other hand, complete denaturation is an absolute requirement for high efficiency PCR. For most applications denaturation times between 30 s and 1 min are recommended. The thermostability of different thermostable DNA polymerases varies considerably: *Pfu* DNA polymerase, for instance, is a highly thermostable enzyme, retaining 94–99% of its polymerase activity after 1 h at 95°C. Unlike *Taq* DNA polymerase, denaturing temperatures up to 98°C can be used successfully with *Pfu* DNA polymerase to amplify GC-rich regions (Chong *et al.*, 1994; Nielson *et al.*, 1995). The Vent DNA polymerase features a half-life of 6.7 h at 95°C and 1.8 h at 100°C. *Taq* polymerase has a half-life of only 40 min at 95°C and 10 min at 97.5°C.

The temperature and length of time required for primer annealing depend upon the base composition (GC content), length and concentration of the amplification primers. The melting temperature (T_m) of a given primer sequence can easily be calculated by the formula:

$$T_m\,(°C) = 2\,(N_A + N_T) + 4\,(N_G + N_C)$$

where N equals the number of primer adenine (A), thymidine (T), guanidine (G) or cytosine (C) bases. Usually, the applied annealing temperature is 5°C below the calculated T_m, but this has to be optimized individually. Generally, one annealing step should last for about 1 min.

The extension time depends on the length of the target sequence, the extension temperature and the type of thermostable DNA polymerase used. For *Taq* and Vent polymerase, extension times of 1 min per kb of expected extension products at 72–75°C are recommended. *Pfu* DNA polymerase needs 2 min per kb at 72°C. Using extension times longer than those calculated is not recommended for DNA polymerases possessing proof-reading exonuclease activity, as this would result in the degradation of primers not yet annealed. Too-short extension times, on the other hand, would decrease the yield of PCR product. To circumvent problems due to the lowered activity of DNA polymerase because of repeated denaturation steps, the extension time can be increased continuously from cycle to cycle. The final extension step is necessary to guarantee that only full length PCR products are obtained. After optimization of all parameters, the optimal number of cycles depends mainly on the starting concentration of the target DNA. Too few cycles gives low product yield, whereas too many cycles can increase the amount and complexity of non-specific background products.

2.3 Amplification from RNA (RT–PCR)

For amplification of RNA, cDNA is first synthesized by reverse transcriptase (RT) and then amplified by PCR as the direct amplification of RNA by commercially available thermostable DNA polymerases is not possible (*Tth* thermostable DNA polymerase has been reported to have reverse transcriptase activity but is not used routinely due to its short half-life; Myers and Gelfand, 1991). In filamentous fungi, RT–PCR is usually applied to generate the corresponding cDNA of a previously cloned genomic fragment used for the confirmation of intron boundaries, and to enable their expression in both bacteria and yeasts.

In a straightforward approach a cDNA fragment may also be directly amplified without previous cloning of the respective genomic clone. This may be necessary if amplification of a desired gene from genomic DNA is unsuccessful because of the presence of an intron in the sequence region used for primer design. Furthermore, the complexity of a reverse-transcribed mRNA pool is lower than that of the complete genome, resulting in a lower background of co-amplified byproducts. This specificity may be further increased by the use of a gene-specific oligonucleotide for reverse transcription of the mRNA and two gene-specific primers for subsequent PCR amplification (also see Section 2.5.3). The oligonucleotide primer for reverse transcription therefore has to be the most 3′-located of the three oligonucleotides.

2.4 RACE: Rapid Amplification of cDNA Ends

The RACE protocol (Frohmann *et al.*, 1988) provides a simple and efficient method for the cloning of cDNA from a gene when the sequence of only a single position in the gene (or protein) is known. The design of the 3′-end primer is based on the predictable sequence of the poly(A)-tail of eukaryotic mRNA (e.g. oligo-dT), and hence individual sequence information is only necessary for the 5′-end. This approach has particular advantages if the sequence of the amino-terminus of a protein can be determined because it bypasses the need to sequence internal peptide fragments of the protein as well (e.g. Marx *et al.*, 1995). The method consists of two steps, described in detail (Fig. 2.1).

The use of oligo-dT primers with a 5′-adaptor sequence containing cleavage sites for several restriction enzymes, preferably enzymes with recognition sites that are found infrequently in genomic DNA such as *Sfi* I, *Not* I, *Mlu* I or *Xho* I, is recommended (McClelland and Nelson, 1987); this facilitates subsequent cloning procedures. The first step consists of a reverse transcription of the mRNA as described earlier. The resulting first strand cDNA is then either purified from RNA by RNase H digestion or used directly for PCR amplification with a gene-specific primer and the oligo-dT primer.

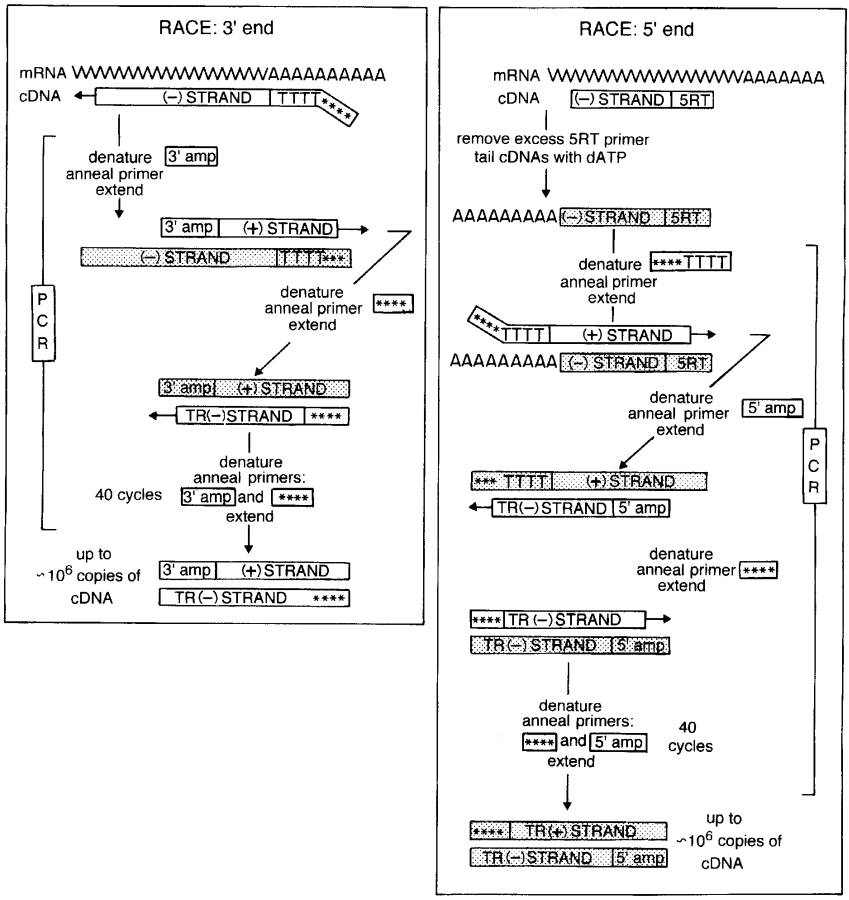

Fig. 2.1. Schematic representation of the RACE protocol. Primers: ****TTTT $(dT)_{17}$-adaptor, containing recognition sequences for restriction enzymes at the 5′-end. 3′amp (amp refers to amplification), specific to gene of interest, complementary to (–) strand. 5RT (RT refers to reverse transcription) and 5′amp, specific to gene of interest, complementary to (+) strand. Open rectangles represent DNA strands actively being synthesized; shaded rectangles represent DNA previously synthesized. At each step the diagram is simplified to illustrate only how the new product formed during the previous step is utilized. A (–) or (+) strand is designated as 'truncated' (TR) when it is shorter than the original (–) or (+) strand, respectively (Frohman *et al.*, 1988).

For the amplification of the 5′-end of cDNA, the mRNA is reverse transcribed using a gene-specific primer. The product of the first strand cDNA synthesis is then subjected to homopolymer (poly(A)) tailing, using terminal polynucleotide transferase. This allows the amplification of cDNA by means of a gene-specific primer at the 3′-end in combination with a homopolymer primer complementary to the tail at the 5′-end of the first

strand cDNA. The specificity and efficiency of the amplification reaction can be increased by using a second gene-specific primer. Again restriction enzyme recognition sequences can be incorporated into the primer sequences to facilitate subsequent cloning strategies.

To obtain a full-length cDNA clone, primers for amplification of 5′ and 3′ fragments must be designed to produce overlapping PCR clones. The two independent clones can be combined to generate a complete cDNA clone by one additional PCR step using primers hybridizing at the 5′-end of the 5′-RACE fragment and at the 3′-end of the 3′-RACE fragment, respectively, using both fragments as templates. Because of the overlapping character of both fragments the internal sequences are used as primers in the first cycles to generate a full-length cDNA clone. This full-length product is further amplified by the primers at the extreme 5′- and 3′-ends of the cDNA. Alternatively, classical cloning procedures can be used to join the two incomplete fragments.

2.5 Specific Applications

2.5.1 Ramp PCR

In Ramp PCR the annealing temperature is lowered from cycle to cycle, usually by 2°C during the first few amplification steps (*PCR Applications Manual*, Boehringer Mannheim, 1995). This can be useful if the annealing temperature of a given primer cannot be exactly determined, for example, because of a high degree of degeneracy. Ramp PCR can also be applied when primers of widely different T_m are used. This is based on the rationale that annealing must be highly specific especially in the first few cycles, whereas later cycles are necessary mainly for further amplification.

2.5.2 Long and accurate PCR (LA-PCR)

Amplification of fragments larger than 5 kb by PCR is usually difficult because of the misincorporation of nucleotides due to premature termination of the extension product (Barnes, 1994a). To overcome this limitation, Barnes employed a mixture of two thermostable DNA polymerases, one that is highly processive, and one exhibiting a 3′ → 5′ exonuclease activity which allows 'proofreading' of the product. A study of different combinations of various enzymes determined that a mixture of 16 parts Klen*Taq* (an exonuclease-free mutant of *Taq* polymerase) to 1 part *Pfu* polymerase, which has a 3′-exonuclease activity, could yield PCR products as large as 35 kb. The mixture was termed Klen*Taq* LA-16 and is commercially available (as are many other optimized combinations of two different thermostable DNA polymerases). The differences between classical PCR and LA-PCR are given in Table 2.3.

Table 2.3. Comparison of conditions for LA-PCR and classical PCR to amplify a 35 kb fragment from λ-DNA (Barnes, 1994b).

LA-PCR	Classical PCR
Klen *Taq*LA-16	Only one enzyme
pH 9.2	pH 8.3
16 mM $(NH_4)_2SO_4$, no KCl	50 mM KCl
50 mM Tris	20 mM Tris–HCl
3.5 mM $MgCl_2$	1–2 mM $MgCl_2$
30–40 s 99°C	60 s 95°C
2 ng λ-DNA	20 ng λ-DNA
33 µl reaction volume	100 µl reaction volume
20 cycles	30 cycles
68°C extension temperature	72°C extension temperature
33 nucleotide primers	20–22 nucleotide primers
11–24 min extension (longer at later cycles)	3–10 min extensions
Hot start or *Taq* antibody start	Cold start, no antibody
Filter tips	Non-filter tips
UVA + 8 MOP[a] before template	No treatment

[a]8 MOP, 8-methoxy-psoralen (for inactivation of contaminating DNA).

2.5.3 PCR with Nested Primers

Even though PCR amplification of a gene with highly degenerate primers should theoretically lead to a million-fold increase in the abundancy of the amplicon over the amount of DNA originally present, the yields may be much lower in practice, and even too low to detect. In many cases the reason for this is the presence of components inhibiting DNA polymerization, which necessitate the dilution of the original DNA solution to a very low level. 'Nested PCR' overcomes this problem by performing a second amplification of the accumulated amplicon using primers annealing within the previously amplified product (nested primers; Albert and Fenyö, 1990). Since the first amplification also reduces the nucleic acid complexity, this method enables a high specificity in the second amplification. Sometimes, only a single internal primer in the second PCR may suffice. Design of a nested primer can be based on the addition of as little as three bases to the 3′-end of the first stage primers. Because of this specificity, nested PCR can be carried out with highly degenerate primers (e.g. 8192-fold; Chen and Suttle, 1995).

2.5.4 Inverse PCR

Inverse PCR allows the amplification of DNA regions located outside of a previously characterized sequence (Ochman *et al.*, 1988; Triglia *et al.*, 1988; Silver and Keerikatte, 1989) and thus enables the direct cloning of a full-

length (e.g. containing also the 5′ and 3′ untranslated regions) gene. This technique is based on the simple rationale that digestion of a given region of DNA with restriction enzymes, and the circularization of the respective fragments before amplification, allows the use of PCR with primers synthesized in the opposite orientations to those normally used for PCR (Fig. 2.2). DNA is cleaved by restriction enzymes which have no recognition site within the gene fragment of interest. DNA fragments are then ligated under conditions that favour the formation of monomeric circles (Collins and Weissman, 1984). The circularized DNA molecules can then be used for PCR amplification either directly or after precipitation of the template DNA by appropriate salts and ethanol. A similar PCR set-up as described for genomic DNA can be used.

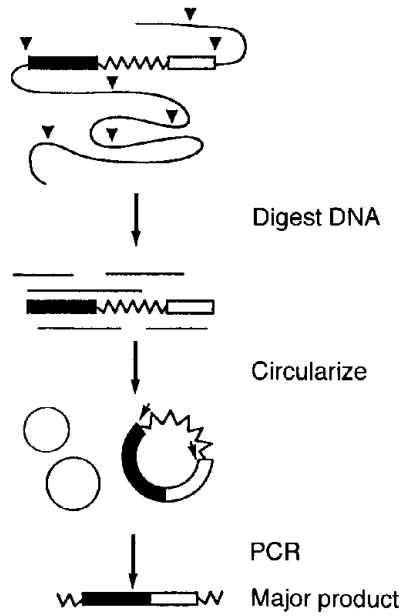

Fig. 2.2. Application of inverse PCR. The core region is depicted as a jagged line. Filled and open boxes represent the upstream and downstream flanking regions, respectively, and restriction enzyme recognition sites are denoted by triangles. Oligonucleotide primers constructed to anneal to the core region and the direction of DNA synthesis are shown by arrows. Figure from Ochman *et al.*, (1990) with permission.

2.5.5 Ligation-mediated PCR (LM-PCR)

Ligation-mediated PCR (LM-PCR; Müller and Wold, 1989) requires the knowledge of only one primer annealing position; the second primer can be defined by ligating a unique DNA linker to it. One of the most important and powerful applications of this method is the rapid cloning of promoters

and other upstream regulatory elements that control the expression of mRNA.

DNA is digested separately with restriction enzymes which generate blunt ends (e.g. *Eco*RV, *Sca*I, *Dra*I, *Pvu*II, *Ssp*I) in order to obtain genomic DNA suitable for addition of the unique linkers. Each batch is then separately ligated to the specially designed adaptor. The desired fragment is then amplified in two steps which are based on the same rationale as nested PCR. In the first step, primers annealing to the linker sequence and to the respective genomic sequence are used. In the second step, the linker primer is used together with another which overlaps with the first 'genomic primer'.

2.6 Cloning of PCR Fragments

Several methods for cloning of PCR-generated DNA fragments have already been published, and only those most routinely used will be described in this chapter. For other methods like ligase-independent cloning (LIC) or uracil DNA-glycosylase (UDG)-treatment of uracil-containing deoxyoligonucleotide primers, the reader is referred to the papers by Aslanidis and De Jong (1990) and Nisson *et al.* (1991). In addition, several new techniques are currently being developed (e.g. regularly published in *Promega Notes*, Promega, Madison, Wisconsin, USA) which renders it impossible to give a complete list of all available methods.

2.6.1 Incorporation of restriction enzyme sites into deoxyoligonucleotide primers

Incorporation of recognition sequences for restriction enzymes into the primers is probably the most widely used method for cloning PCR fragments. The main advantage of this method is that the fragment can be cloned into a vector construct at precisely the location desired. Any site not contained within the fragment itself can be incorporated into the primer design. When amplifying an unknown sequence, restriction sites should be chosen which occur infrequently in the genome, such as *Not*I, *Sfi*I, *Mlu*I or *Xho*I; in addition, recognition sequences for several restriction enzymes can be incorporated to minimize the chance of cloning an uncomplete PCR fragment because of an internal restriction site.

To guarantee the direction of the cloned insert, different restriction target sites on each primer are usually used. Adding bases to the 5′-end of the primer is the simplest approach and has no effect on the PCR reaction (Scharf *et al.*, 1986; Kaufman and Evans, 1990). When creating a restriction site by addition of sequences to the 5′-end of a primer, it is important to consider that restriction enzymes are endo- (not exo-) nucleases and thus do not usually cleave at the end of a nucleotide strand. Hence the included restriction site needs to be prolonged by the addition of several bases. To achieve efficient cleavage, three

additional nucleotide bp are generally sufficient to act as an annealing clamp. However, some enzymes, for example *Not* I, need at least 10 bases of double-stranded DNA to restrict at its recognition sequence.

One drawback to restriction enzyme site incorporation is the inability to achieve efficient cleavage. Inhibition of digestion can occur because of the presence of incompatible polymerase buffers and the molar excess of primers left over from the PCR reaction. Some restriction enzymes are inhibited by restriction site-containing primers (Blanck *et al.*, 1995) and therefore, it is often necessary to purify the fragment before restriction enzyme treatment (Table 2.4).

Table 2.4. Activities of restriction enzymes in a PCR mix.

Restriction enzyme	% activity in PCR mix	Restriction enzyme	% activity in PCR mix
*Apa*I	100	*Mlu*I	<5
*Asp*700	10	*Nae*I	0
*Asp*718	100	*Nco*I	50
*Avi*II	30	*Not*I	0
*Bam*HI	100	*Nru*I	75
*Bbr*PI	100	*Pst*I	90
*Bfr*I	100	*Pvu*I	<5
*Bgl*II	0	*Pvu*II	100
*Cla*I	100	*Sac*I	100
*Dra*I	100	*Sal*I	0
*Ecl*XI	0	*Sca*I	<5
*Eco*47III	0	*Sma*I	100
*Eco*RI	50	*Sna*BI	50
*Eco*RV	10	*Sph*I	<5
*Hin*dIII	10	*Ssp*I	0
*Hpa*I	100	*Stu*I	30
*Kpn*I	50	*Xha*I	60
*Ksp*I	0	*Xho*I	>5
*Mam*I	20		

Relative activities of restriction enzymes in a PCR mixture (10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM dNTPs, pH 8.3 at 20°C) compared with activity under optimal conditions (SuRE/Cut buffer Boehringer Mannheim) (Blanck *et al.*, 1995).

2.6.2 T/A cloning

T/A-cloning relies on the terminal deoxynucleotide transferase activity of some of the polymerases used in PCR. Clark (1988) showed that some DNA

polymerases and reverse transcriptases contain a terminal deoxynucleotidyl transferase (TdT) activity that results in the addition of one or more nucleotides at the 3′-ends of blunt-ended DNA molecules, which is both nucleotide and polymerase specific (Hu, 1993); e.g., *Taq* DNA polymerase extends a single dG nucleotide if the 3′-terminal nucleotide on the fragment is a dG but adds a dA if the 3′-terminal nucleotide is a dC. A 3′-terminal dT nucleotide results in the non-addition of a dT and the addition of a dA. Fragments ending with a dA had an additional dA added to the 3′-ends with extremely low efficiency by polymerases that were found to have the TdT activity; there appears to be no consistent patterns for which bases were added. *Pfu* DNA polymerase does not show any TdT activity and so this polymerase cannot be used for amplification of fragments for subsequent cloning.

The vector into which dA-tailed PCR fragments are to be cloned must contain a 3′-T overhanging sequence. This can be obtained either by incubating a blunt-ended vector with *Taq* DNA polymerase and an excess of dTTP, or by incubating a blunt-ended vector with dideoxythymidine-triphosphate (ddTTP) and terminal transferase (Holton and Graham, 1990); the use of ddTTP ensures the addition of a single dT residue only.

2.6.3 Blunt-end cloning

As with the T/A System, blunt-end cloning does not require the addition of extra bases to the primer sets. PCR-fragments generated by *Taq* polymerase or any other polymerase adding a non-templated nucleotide at the 3′-end must be treated with Klenow, T4 or *Pfu* polymerase to generate blunt ends. The vector must also be blunt-ended (e.g., by cutting with *Eco*RV or *Sma*I). To improve the efficiency of blunt-end cloning, the use of an optimized blunt-end ligation buffer is recommended. The direction of fragments after blunt-end cloning is usually not known, but this drawback can be circumvented if one of the primers is phosphorylated at the 5′-end, and the other one is not and therefore still contains the 5′-OH. The vector is cut with the first restriction enzyme, then dephosphorylated and afterwards cut with the second restriction enzyme to create two different blunt ends; one with a 5′-OH, the other with a 5′-phosphate. The ligation of the one-sided-phosphorylated PCR-fragment into the one-sided-dephosphorylated vector allows unidirectional cloning.

Religation of the vector can be a major problem with blunt end cloning of PCR-fragments, but this can be avoided by the use of the blunt-end-generating restriction endonuclease *Srf* I together with an appropriate vector. The recognition sequence of *Srf*I is an 8 bp palindrome. Since it is very unlikely that such a sequence occurs in the unknown sequence of the gene of interest, *Srf*I can be added to the ligation mixture where it cleaves any religated vector (Simcox *et al.*, 1991; Liu and Schwartz, 1992).

Table 2.5. Examples of fungal genes cloned by PCR.

Organism	Reference	Gene name	Primer		Primer design based on		
			*dp	ndp	aa/seq	aa/c	dna/c
Aspergillus							
A. niger	Inoue *et al.*, 1991	Proteinase A	•		•		
A. niger	Ehrlich *et al.*, 1993	*phyB* (phytase gene)		•	•		
A niger	Jarai *et al.*, 1994	*pepD* (subtilisin-like protease)	•			•	
A. niger	Van den Brink *et al.*, 1995	*cprA* (NADPH cytochrome P450 oxidoreductase)	•			•	
A. oryzae	Gomi *et al.*, 1993	*pepA* (acid protease)	•		•		
A. oryzae	Lee *et al.*, 1995	*nucS* (nuclease S1)	•		•		
A. fumigatus	Sirakova *et al.*, 1994	Metalloproteinase	•		•		
A. fumigatus	Reichard *et al.*, 1995	*pep* (aspatic proteinase)		•			•
A. flavus, A. fumigatus	Ramesh *et al.*, 1995	*mep20* (metalloproteinase)	•			•	
A. parasiticus	Feng and Leonard, 1995	*pksL1* (polyketide synthase)	•			•	
A. parasiticus	Cary *et al.*, 1995	*pecA* (polygalacturonase)		•			•
Neurospora							
N. crassa	Yajima *et al.*, 1991	Photolyase gene	•			•	
N. crassa Mauriceville	Young and Marzluf, 1991	*nmr* (negative-acting nitrogen control gene)		•			•
N. crassa	Stone *et al.*, 1993	*gla-1* (glucoamylase)	•			•	
N. crassa	Tao and Chen, 1994	*elF*-5A (initiation factor 5A)	•			•	
N. crassa	Yatzkan and Yarden, 1995	*pph-1* (2A protein phosphatase)	•			•	
N. crassa	Maier *et al.*, 1995	GTP-cyclohydrolase I gene	•			•	
N. intermedia	Young and Marzluf, 1991	*nmr* (negative-acting nitrogen control gene)		•			•

Continued overleaf

Table 2.5. continued.

Organism	Reference	Gene name	Primer		Primer design based on		
			*dp	ndp	aa/seq	aa/c	dna/c
N. sitophila	Young and Marzluf, 1991	*nmr* (negative-acting nitrogen control gene)		•			•
Penicillium							
P. chrysogenum	Marx *et al.*, 1995	*paf* (antifungal protein)	•		•		
P. chrysogenum	Haas *et al.*, 1995	*nre* (nitrogen regulatory protein)	•			•	
P. camembertii U-150	Yamaguchi *et al.*, 1991	*mdlA* (mono- and diacylglycerol lipase)	•		•		
Trichoderma							
T. reesei	Barnett *et al.*, 1991	*bgl1* (β-glucosidase)	•		•		
T. reesei	Törrönen *et al.*, 1992	*xyn1, xyn2* (xylanase I and II)	•		•		
T. reesei	Strauss *et al.*, 1995	*cre1* (carbon catabolite repressor protein)	•			•	
T. longibrachiatum	González *et al.*, 1992	*egl1* (endoglucanase I)		•			•
T. harzianum	Heidenreich and Kubicek, 1994	*pyr4* (ornithine-5′-phosphate decarboxylase)		•			•
Others							
Aureobasidium pullulans	Li and Ljungdhal, 1994	*xynA* (xylanase A)	•		•		
Amanita muscaria	Kreuzinger *et al.*, 1996	*gpd* (glyceraldehyde-3-phosphate dehydrogenase)		•			•
Boletus edulis	Kreuzinger *et al.*, 1996	*gpd* (glyceraldehyde-3-phosphate dehydrogenase)		•			•
Boletus edulis	Mehmann *et al.*, 1994	*BoeCHS1* (chitin synthase)	•			•	
Botrytis cinerea	Causier *et al.*, 1994	*chs1* (chitin synthase)	•			•	
Cantharellus ciarius	Mehmann *et al.*, 1994	*CacCHS1* (chitin synthase)	•			•	
Ceriporiopsis subvermispora	Rajakumar *et al.*, 1996	*lip* (lignin peroxidase)	•				•

Cochliobolus carbonum	Scott-Craig *et al.*, 1990	*PGN1* (endopolygalacturonase)	•		•		
Cochliobolus carbonum	Nikolskaya *et al.*, 1995	*cv-2*, *cv-7*, *cv-19* (CPS, cyclic peptide synthase)	•			•	
Cochliobolus carbonum	Murphy and Walton, 1996	*alp1* (serin protease)	•		•		
Cortinarius odorifer	Mehmann *et al.*, 1994	*CooCHS1*, *CooCHS2* (chitin synthase)	•			•	
Cylindrocladium macrosporum	Nikolskaya *et al.*, 1995	*cyl-1*, *cyl-2*, *cyl-3* (CPS, cyclic peptide synthase)	•			•	
Diheterospora chlamydosporia	Nikolskaya *et al.*, 1995	*dih-1* (CPS, cyclic peptide synthase)	•			•	
Elaphomyces muricatus	Mehmann *et al.*, 1994	*ElmCHS1*, *ElmCHS2* (chitin synthase)	•			•	
Fusarium solani	Gonzales-Candelas and Kolattukudy, 1992	*pelA* (pectate lyase)	•		•		
Fusarium oxysporum	Sheppard *et al.*, 1994	*Bfam 1*, *Cfam 1*, *Cfam 2*, *Ffam 1*, *Kfam 1* (cellulase family-specific proteins)	•			•	
Hebeloma crustuliniforme	Mehmann *et al.*, 1994	*HecCHS1* (chitin synthase)	•			•	
Hebeloma mesophaeum	Mehmann *et al.*, 1994	*HemCHS1* (chitin synthase)	•			•	
Lactarius deterrimus	Mehmann *et al.*, 1994	*LcdCHS1* (chitin synthase)	•			•	
Lactarius deterrimus	Kreuzinger *et al.*, 1996	*gpd* (glyceraldehyde-3-phosphate dehydrogenase)		•			•
Laccaria laccata	Mehmann *et al.*, 1994	*LalCHS1*, *LalCHS2* (chitin synthase)	•			•	
Orpinomyces sp.	Chen *et al.*, 1995	*cypB* (cyclophilin)	•		•	•	
Phanerochaete sordida	Rajakumar *et al.*, 1996	*lip* (lignin peroxidase)	•				•
Phycomyces blakesleeanus	Maier *et al.*, 1995	GTP-cyclohydrolase I gene	•			•	
Pleurotus ostreatus	Giardina *et al.*, 1995	*pox1* (phenol oxidase)	•		•		
Russula adulterina	Mehmann *et al.*, 1994	*RuaCHS1* (chitin synthase)	•			•	
Rhizopogon vulgaris	Mehmann *et al.*, 1994	*RhvCHS1*, *RhvCHS2* (chitin synthase)	•			•	
Tuber uncinatum	Mehmann *et al.*, 1994	*TuuCHS1* (chitin synthase)	•			•	
Xerocomus badius	Mehmann *et al.*, 1994	*XebCHS1* (chitin synthase)	•			•	

*dp, Degenerate primers; ndp, non-degenerate primers; aa/seq, primer design based on partial amino acid sequences of the purified protein; aa/c, primer design based on one or more amino acid sequences from related proteins from other species; dna/cl, primer design based on one or more DNA sequences from related genes from other species.

2.7 Conclusions

The availability of a range of strategies for PCR cloning has revolutionized the cloning of genes from filamentous fungi. Table 2.5 shows a subset of the data available from Medline summarizing genes cloned by some of the PCR strategies outlined in this chapter. While this list is certainly not complete, we have tried to show representative examples for a wide variety genes, indicating how strongly PCR has taken over fungal gene cloning. It is also apparent from Table 2.5 that there has been a strong increase in the number of publications in recent years, indicating that PCR cloning has now become an established tool in fungal molecular genetics. In addition (and not shown here), more sophisticated techniques such as differential display and ligation-mediated PCR are being applied increasingly to filamentous fungi. It is anticipated that these advances in methodology will help raise fungal mycology to the status already reached by the molecular genetics of unicellular pro- and eukaryotes.

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