PP-RACE* as a “new” variant of the RACE strategy

*(RACE = Rapid Amplification of cDNA Ends)

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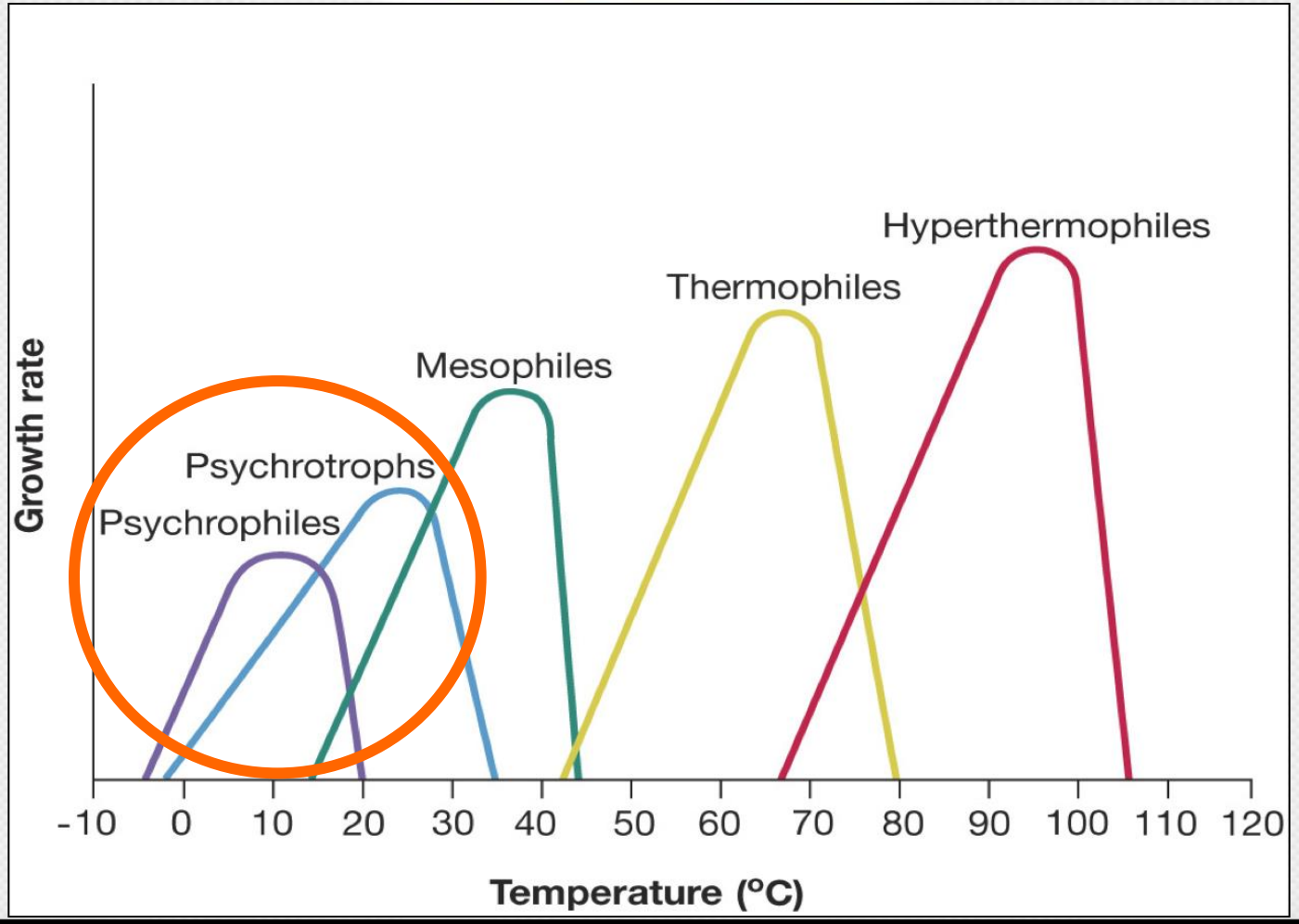
The goal of my thesis

- **Searching for novel cold-active hydrolases with biotechnological potential**

Temperature preference and tolerance of organisms



Biotechnological potential of cold-active enzymes

- Advantages:

  Energy savings, minimizing of undesirable products synthesized at higher temperatures, heat inactivation

- Applications in many fields

detergents


molecular engineering

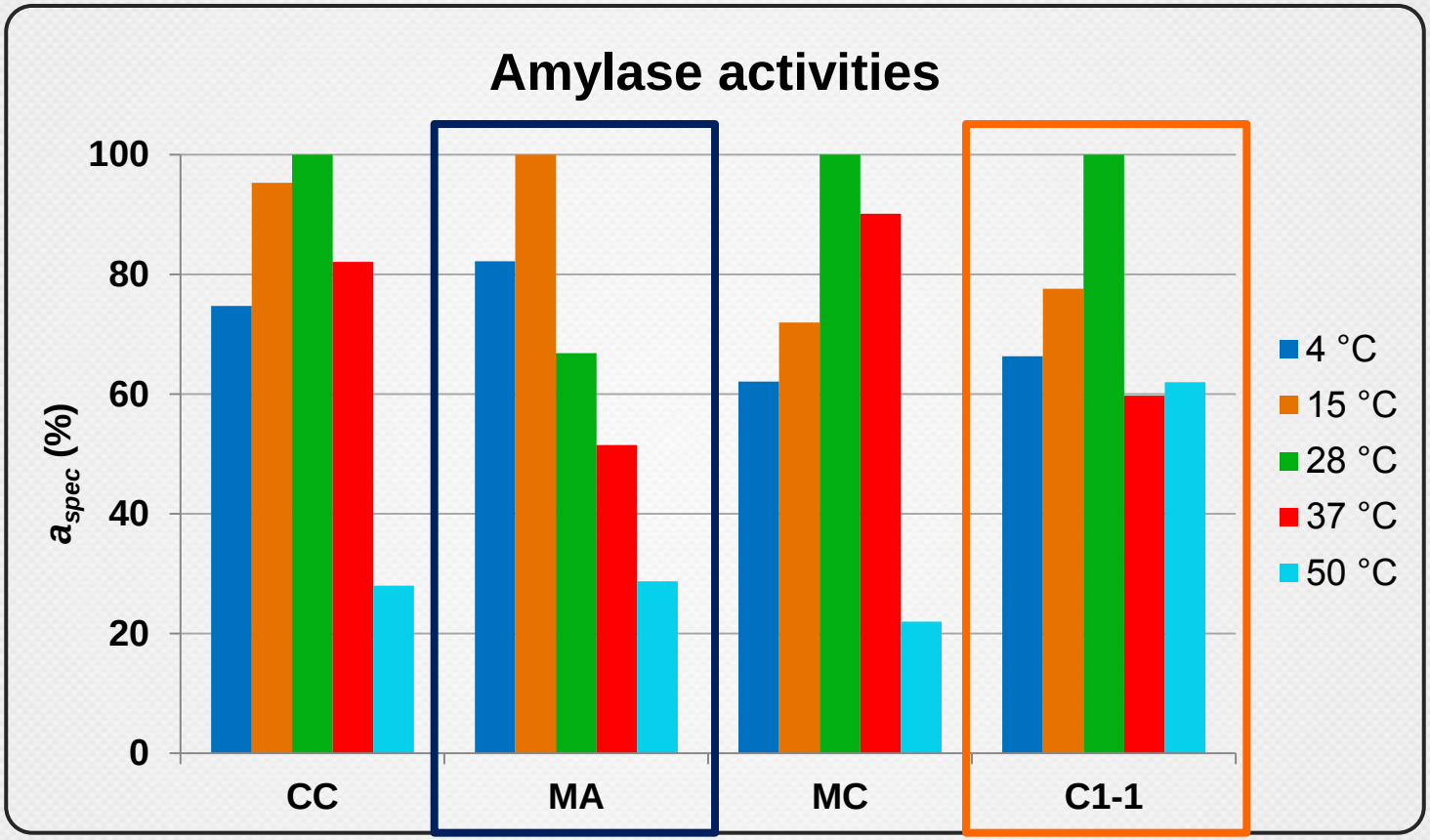

food industry


textile industry

Previous work

- Screening of amylase and cellulase activities in cold-adapted bacteria or yeasts using agar plates
- Measuring of temperature profiles of these activities in lysates using dinitrosalicylic acid

Temperature profiles



Genes from yeasts

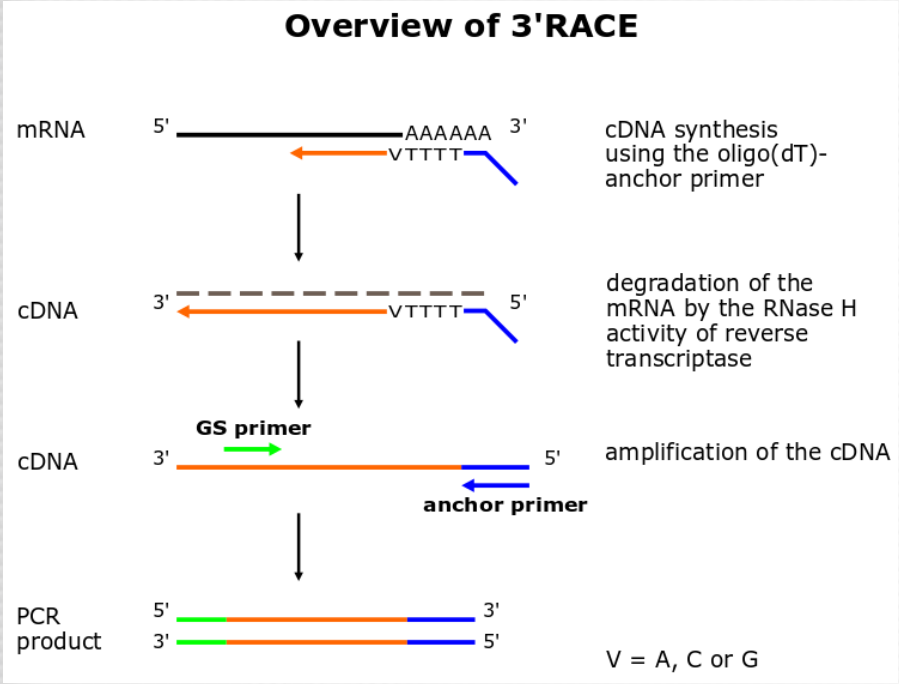
- *Mrakiella aquatica* – amylase (MA)

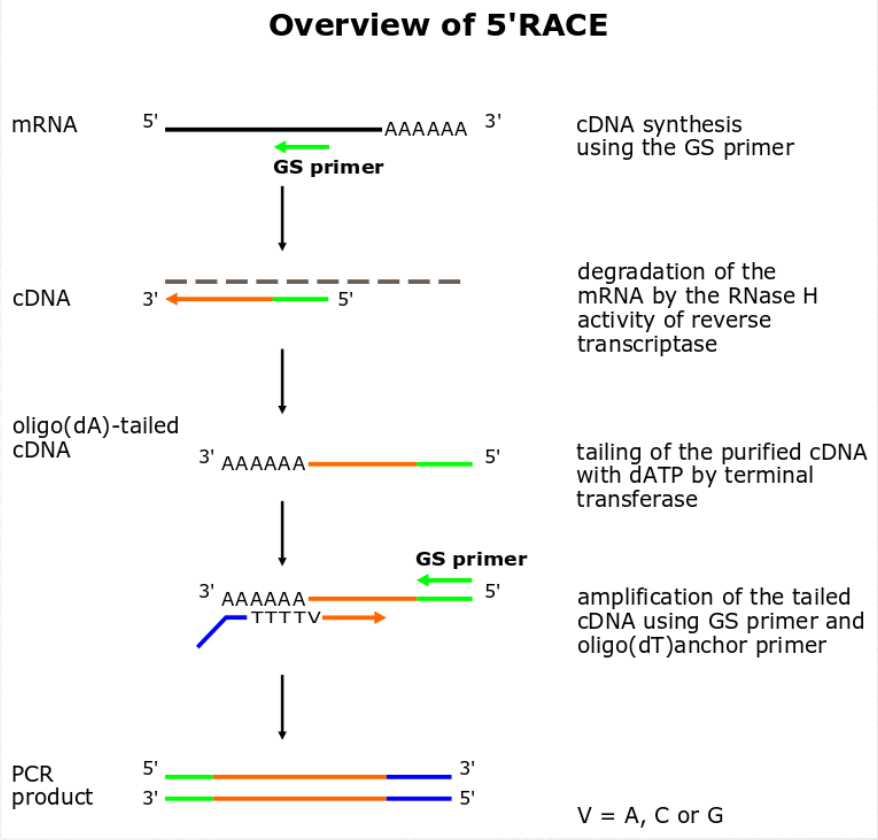
- Design of gene-specific primers
- Isolation of total RNA from yeasts

- **RACE methods**

3‘/5‘ RACE overview

- **3’/5’ RACE method**

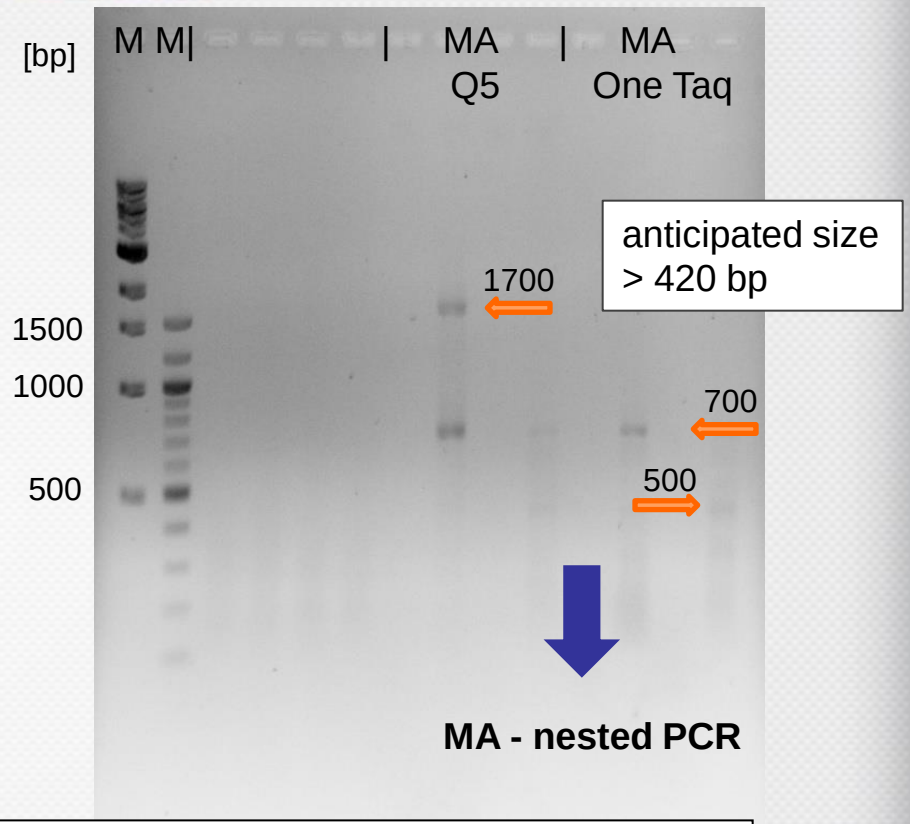




3’ RACE

PCR

- **gene-specific primer Amy1 Mrak FW and anchor2 primer**
- **template = cDNA (from RT with oligo(dT)anchor2 primer)/ reaction mixture from previous PCR**
- **different amounts and type of template**
- **Q5 / One Taq Hot Start polymerase**
- **annealing temperature gradients**



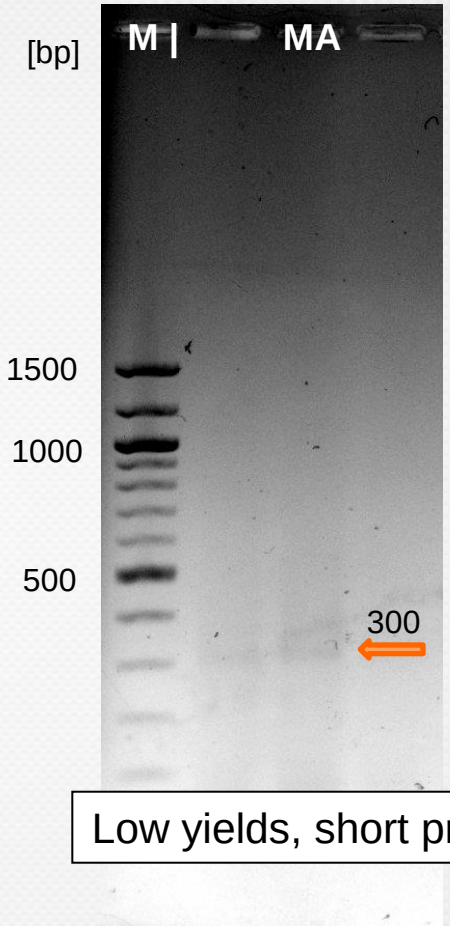
Isolation of the products from gel and sequencing:

- **700 bp – verified by sequencing**

5’ RACE

PCR

- **gene-specific primer Amy1 Mrak REV or Cel1 Cryp REV, anchor2 primer and oligo(dT)anchor2 primer**
- **template DNA cDNA (from RT using gene-specific primer) / reaction mixture from previous PCR**
- **different amounts of template**
- **Q5 / One Taq Hot Start polymerase**
- **annealing temperature gradients**



Low yields, short products, smear

Searching for another method

1558–1560 *Nucleic Acids Research, 1999, Vol. 27, No. 6*

Amplification of cDNA ends based on template-switching effect and step-out PCR

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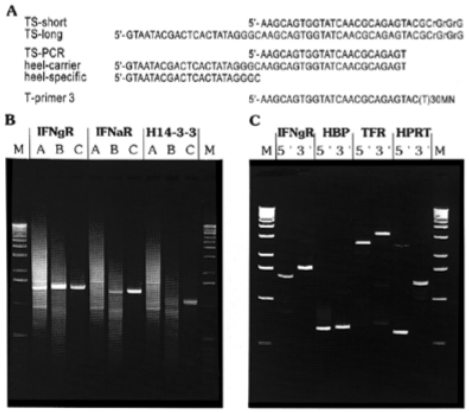
ABSTRACT

A new method for amplifying cDNA ends is described which requires only first-strand cDNA synthesis and a single PCR to generate a correct product with very low or no background. The method can be successfully applied to total RNA as well as poly A+ RNA. The same first-strand cDNA can be used to amplify flanking sequences of any cDNA species present in the sample.

The PCR-suppression effect (PS-effect; 1,2) has promoted the development of several novel techniques for gene identification and investigation. The basic principle is that DNA molecules flanked by inverted terminal repeats ~40 bp long ('suppression sequences') cannot be amplified with a primer corresponding to the distal half of the repeat.

Here we propose a new convenient way of introducing suppression sequences, which we call step-out PCR (SO-PCR). A similar idea has recently been employed to overcome a very specific problem: primer-dimer amplification (3). In our view, it has a wider area of application: it can be utilized whenever there



Searching for another method

- MMLV reverse transcriptase
  - able to add a few non/template nucleotides (mostl C) to the 3‘-end of a newly synthesized cDNA strand

Searching for another method

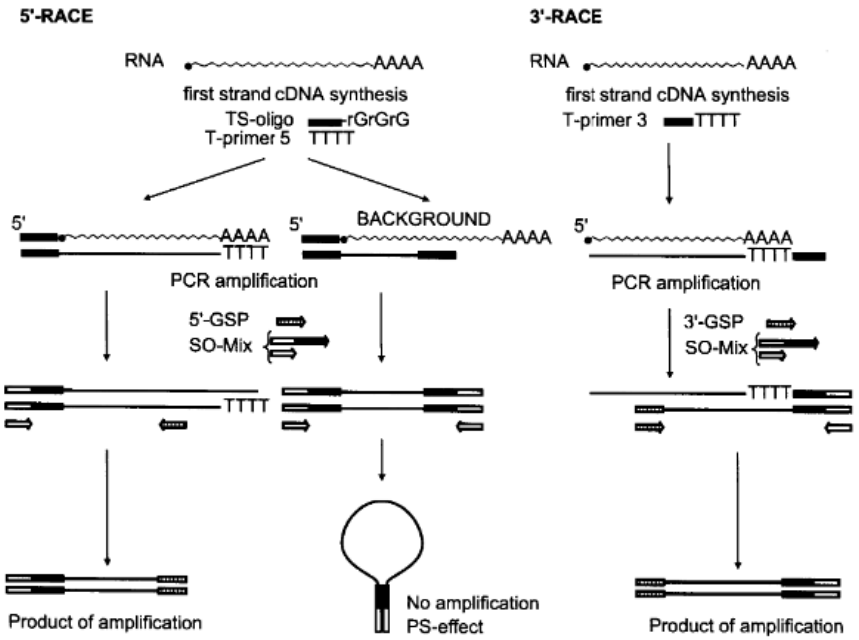
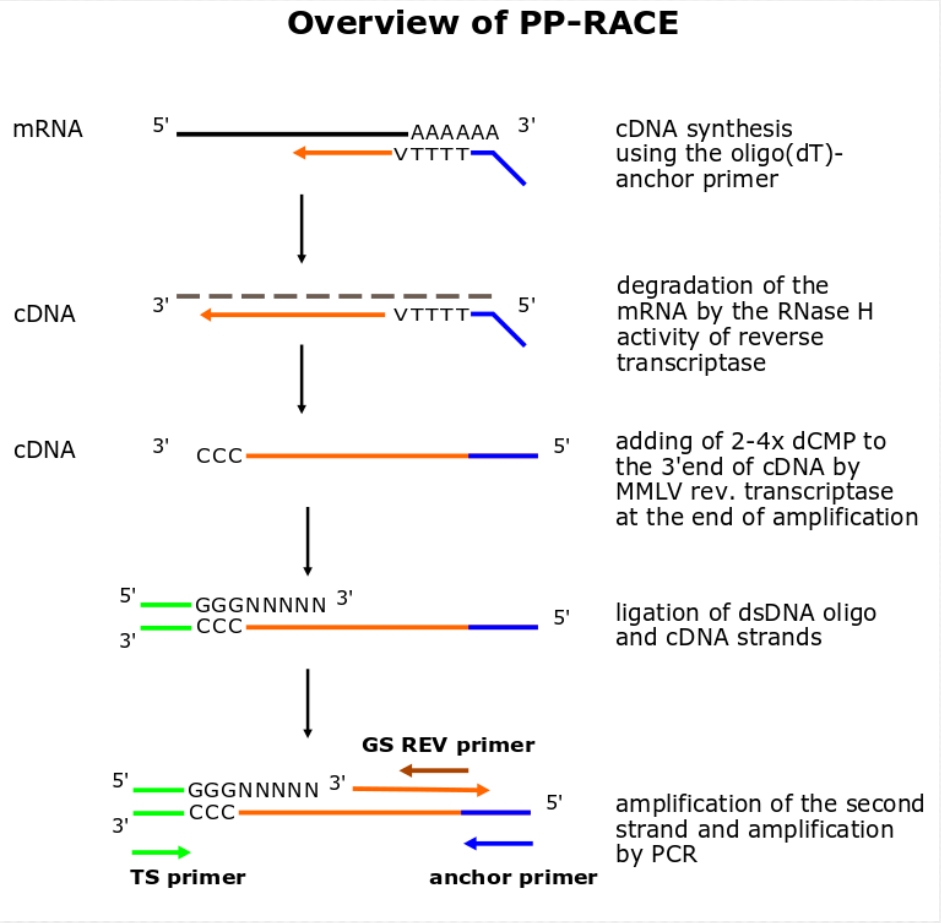


Figure 2. Schematic representation of 5′- and 3′-step-out RACE. Two separate RT-reactions were carried out for 5′-RACE and for 3′-RACE. Each contained 0.5 µg of poly A+ or 1 µg of total RNA, 50 mM Tris–HCl pH 8.3, 75 mM KCl, 6 mM $MgCl_2$, 2 mM DTT, 1 mM each dNTP and 200 U of MMLV reverse transcriptase (SuperScript II, Life Technologies), in a total volume of 10 µl. In addition, the 5′-RACE RT reaction contained 0.5 µM of T-primer '5' (oligo dT stretch 30 bp long) and 0.5 µM of template-switching oligo (TS-short, see Fig. 1A), and the 3′-RACE reaction contained 0.5 µM of T-primer 3 (see Fig. 1A). The reactions proceeded for 90 min at 42°C. Then they were diluted in water (20-fold when polyA+ RNA is used and 10-fold for total RNA), and 1 µl of these dilutions was put into 20 µl of PCR mixture. PCR mixtures for 5′- and 3′-reactions were the same except for the gene-specific primer. They contained 1× Advantage KlenTaq Polymerase Mix with provided buffer (Clontech), 200 µM dNTPs, 0.15 µM of gene-specific primer, 0.02 µM of 'heel-carrier' oligo and 0.15 µM of 'heel-specific' oligo (see Fig. 1A). Gene-specific primers were from commercially available amplimer sets (Clontech). In our study, we used touchdown PCR (6). The cycling profile was (Perkin-Elmer 480 thermocycler): 5 cycles for 30 s at 94°C; 2.5 min at 72°C; 5 cycles for 30 s at 94°C; 2.5 min at 70°C; 25–35 cycles (depending on the transcript's abundance) for 30 s at 94°C; 2.5 min at 68°C. All oligonucleotides were purified through polyacrylamide gel before use.

PP-RACE overview



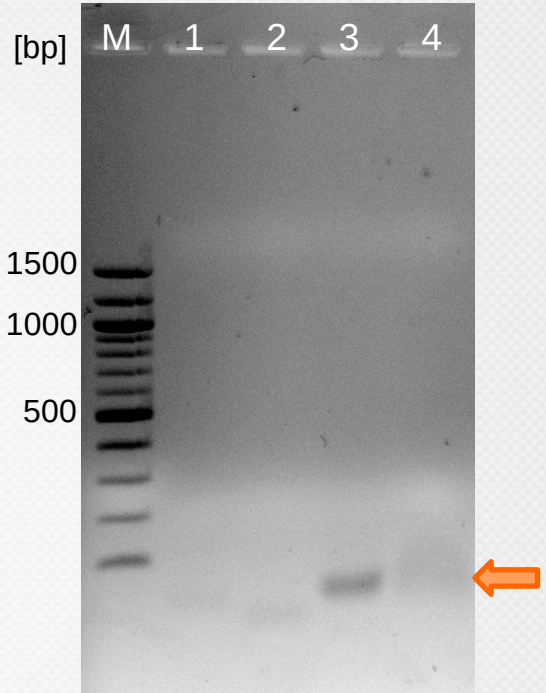
PP-RACE overview

STEP 1

- **Phosphorylation of 5' end of TS oligo complement**

STEP 2

- **Hybridization of TS oligo and TS oligo complement = dsDNA oligo**
- **Control = restriction cleavage by EcoRI**



M – 100 bp marker
1 – TS oligo*, size = 43 nt
2 – TS oligo complement*, size = 35 nt
3 – dsDNA oligo*
4 – dsDNA oligo after the cleavage*

**100 ng each*

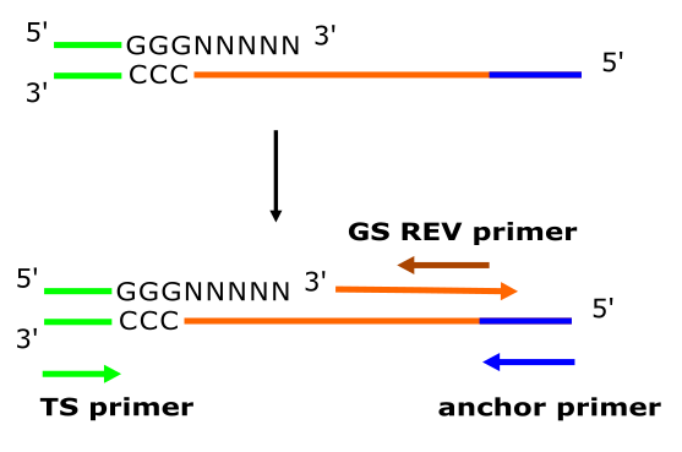
PP-RACE– results

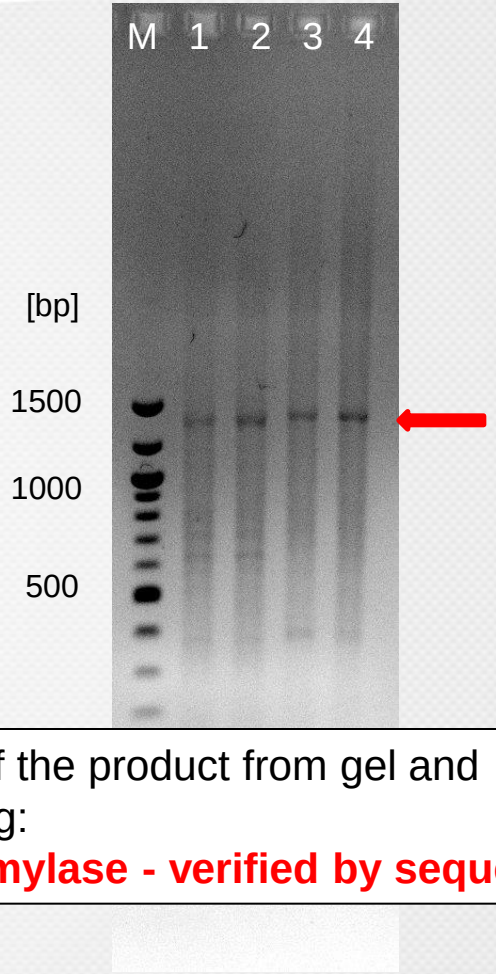
STEP 3

- **Ligation of dsDNA oligos and cDNA and “CleanUp” of ligated DNA products**

STEP 4

- **Amplification using TS primer and GS REV primer → reaction mixture after PCR served as a new template for another PCR**



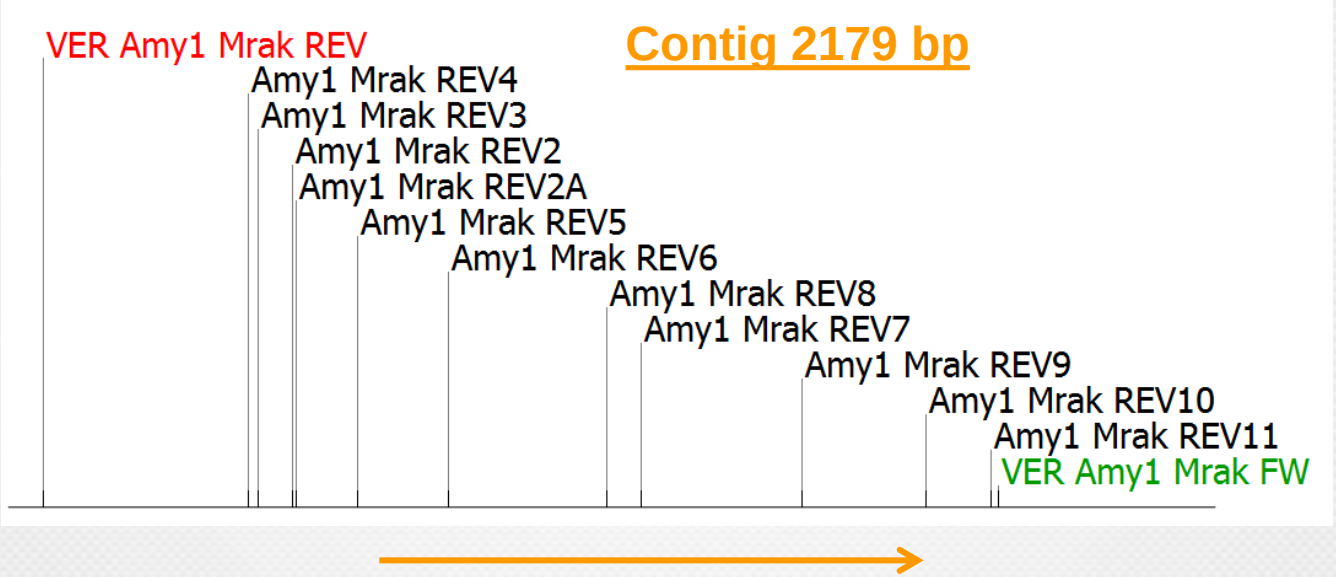


Isolation of the product from gel and sequencing:

- **Amylase - verified by sequencing**

PP-RACE– results

Design of new GS primers and sequencing – step by step



Mrakiealla aquatica Amylase

Expasy – Translate tool – searching for ORFs

5'3' Frame 2

```
LEINLPLHSVQMEEGCRQSTSEQPDNRWLGMELKCMSCFVKLVYLQPVQEGTGR-CGLTP
QMLKARNHQSAKLVGPTRGVRASHRQGYSI-QDTKTDIIKDVDVIK-NNLDMTVARN-IE
LVSL-LALPTLPSS-PIMPPTDPKGETGAFSAEKAAHPEITSVPANFTMIQFFEWYAEGG
GVHWKKFEERSKSLGEMGITAAWLPPPTKGSSTEGTGYDVYDLYDLGEFDQKGNTRTNWG
TKEELVSAIKTARENGVISYIDAVLNHKAGADKTETFMATEVDSDDRNKEISGMYNIDGW
TGFEFPGRGDKYSSFKWGYNHFTGVDYDAKNEKTSIFKIHGDGKTWAKAVDGENGNYDYL
MFADIDHDHPDVEKDINDWGVWVIKETGAEGFRFDAIKHIDRGFISQFVQHVRKEVGNDK
MFCVGEFWKDSLDALNKYTDSLGTQFSVFDTPLHYNFKEAGEGGNDYDMRKIFDGTVVQT
NPIDAVTLVDNHDTQCGQSLESWVNPQFKPLAYSLILLRVDGYPCIFYGDLYGCGGRTRR
SPCPSWTTSSAAESGSRTESSETTGTTPTASDGSEWETTSTTDAPSSSRTEGMDRRGWSS
GRRRRGRSTPTSSGGSRARSSWERMGGPSSPATLCRCRSGRRRTRVSVRRSTRTRSARY-
TGRGGGPGGRQRLSRPFSLSLSLSLSLSFLSLSRARRSLCWIGLGPTHTRERHRGGPSRA
QAPTKN
```

Mrakiealla aquatica Amylase

blastp

Conserved domains on [lcl|Query_42752]

View: Standard Results

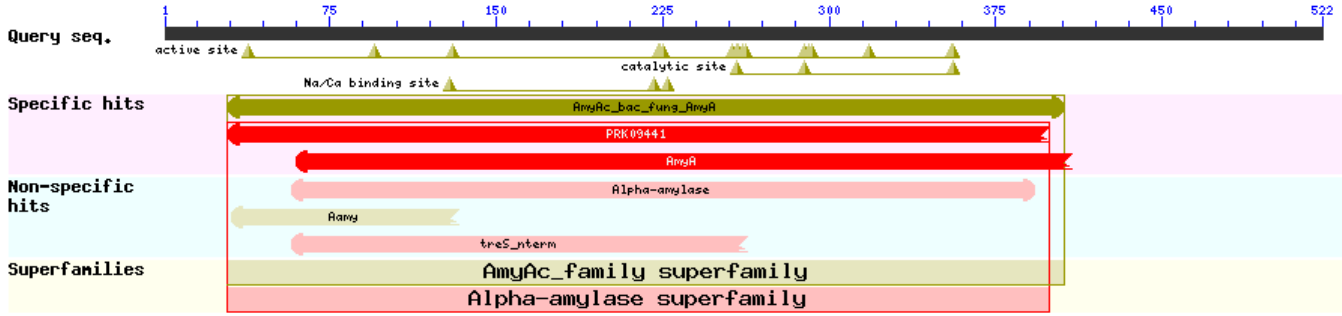
Local query sequence

Protein Classification

alpha-amylase (domain architecture ID 10183071)
bacterial and fungal alpha amylase catalyzes the hydrolysis of alpha-(1,4) glycosidic linkages of glycogen, starch, related polysaccharides, and some oligosaccharides.

Graphical summary

Zoom to residue level | show extra options »



Search for similar domain architectures | Refine search

List of domain hits

Name	Accession	Description	Interval	E-value
[+] AmyAc_bac_fung_AmyA	cd11318	Alpha amylase catalytic domain found in bacterial and fungal Alpha amylases (also called 1, ...	29-406	0e+00
[+] PRK09441	PRK09441	cytoplasmic alpha-amylase; Reviewed	29-399	0e+00
[+] Alpha-amylase	pfam00128	Alpha amylase, catalytic domain; Alpha amylase is classified as family 13 of the glycosyl ...	57-393	1.47e-18
[+] Aamy	smart00642	Alpha-amylase domain;	30-133	3.20e-18
[+] AmyA	COG0366	Glycosidase [Carbohydrate transport and metabolism];	59-409	3.74e-17
[+] treS_nterm	TIGR02456	trehalose synthase; Trehalose synthase interconverts maltose and alpha, alpha-trehalose by ...	57-263	5.88e-04

Blast search parameters

Data Source: Live blast search **RID** = 67V2SNUZ01N
User Options: **Database:** CDSEARCH/cdd v3.16 **Low complexity filter:** no **Composition Based Adjustment:** yes **E-value threshold:** 0.01 **Maximum number of hits:** 500

Original method

Nucleic Acids Research, 1999, Vol. 27, No. 21 e31

CapSelect: A highly sensitive method for 5′ CAP-dependent enrichment of full-length cDNA in PCR-mediated analysis of mRNAs

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Received July 19, 1999; Revised and Accepted September 17, 1999

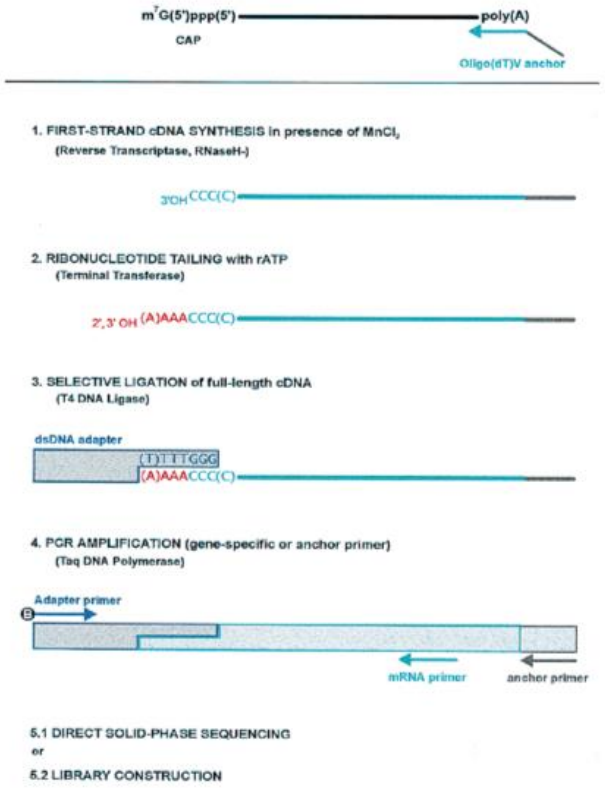
ABSTRACT

Here we present CapSelect as a novel experimental approach for the selective enrichment of full-length cDNAs in PCR-mediated analysis of mRNA sequences. The method combines the 5′-CAP-dependent addition of specifically three to four non-templated dCMP residues to the 3′-end of full-length cDNAs by reverse transcriptases in the presence of manganese and the controlled ribonucleotide tailing of cDNA ends by terminal deoxynucleotidyl transferase using rATP. By virtue of the generated terminal sequence motif (5′-$dC_{3-4}rA_{3-4}$), full-length cDNAs are selectively anchored to a double-stranded DNA adapter (with a dT_{3-4} dG_3 3′-overhang) by *T4* DNA ligase. The technique described is highly efficient, discriminates premature termination products and enriches full-length cDNAs.

Generation of full-length cDNAs from an mRNA template is a major challenge in biotechnology research. It constitutes the ultimate prerequisite in the construction of cDNA libraries, expression profiling and as a new area in DNA chip technology. Utilising current techniques, the 5′-ends of genes tend to be under-represented in cDNA populations, especially if a premature termination products generated in cDNA synthesis. The CapFinder™ method (7–9) offers a solution to this central problem and also allows the analysis of limited starting material. The technique relies on the terminal transferase and the template switching activity of the reverse transcriptases. The addition of cytosine residues to the 3′-end of full-length cDNAs allows the reverse transcriptase to generate a specific anchor sequence complementary to a template switching oligonucleotide. However, it should be stressed that the nucleotide addition activity of reverse transcriptases pausing at the 7-methylguanosine cap structure of the mRNA template has not been studied in detail. Moreover, structural requirements of the mRNA template and/or the cDNA terminus for template switching activity can lead to inefficient amplification and therefore to a biased representation within the amplified cDNA pool. Another problem associated with this technique, background amplification produced by a single primer, was recently shown to be overcome by step-out PCR utilising the PCR-suppression effect (10).

Here, we present CapSelect (Fig. 1) as a novel technique for the selective enrichment of full-length cDNAs. The method was successfully applied for the specific enrichment of complete mRNA 5′-ends in PCR-mediated sequence analysis, cDNA library construction and direct transcription start site mapping.

Original method



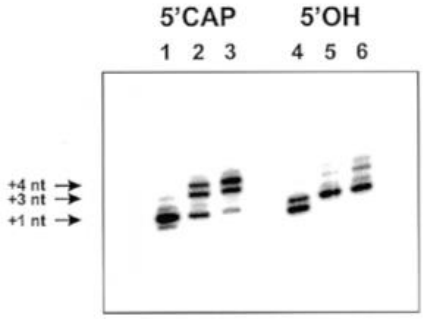


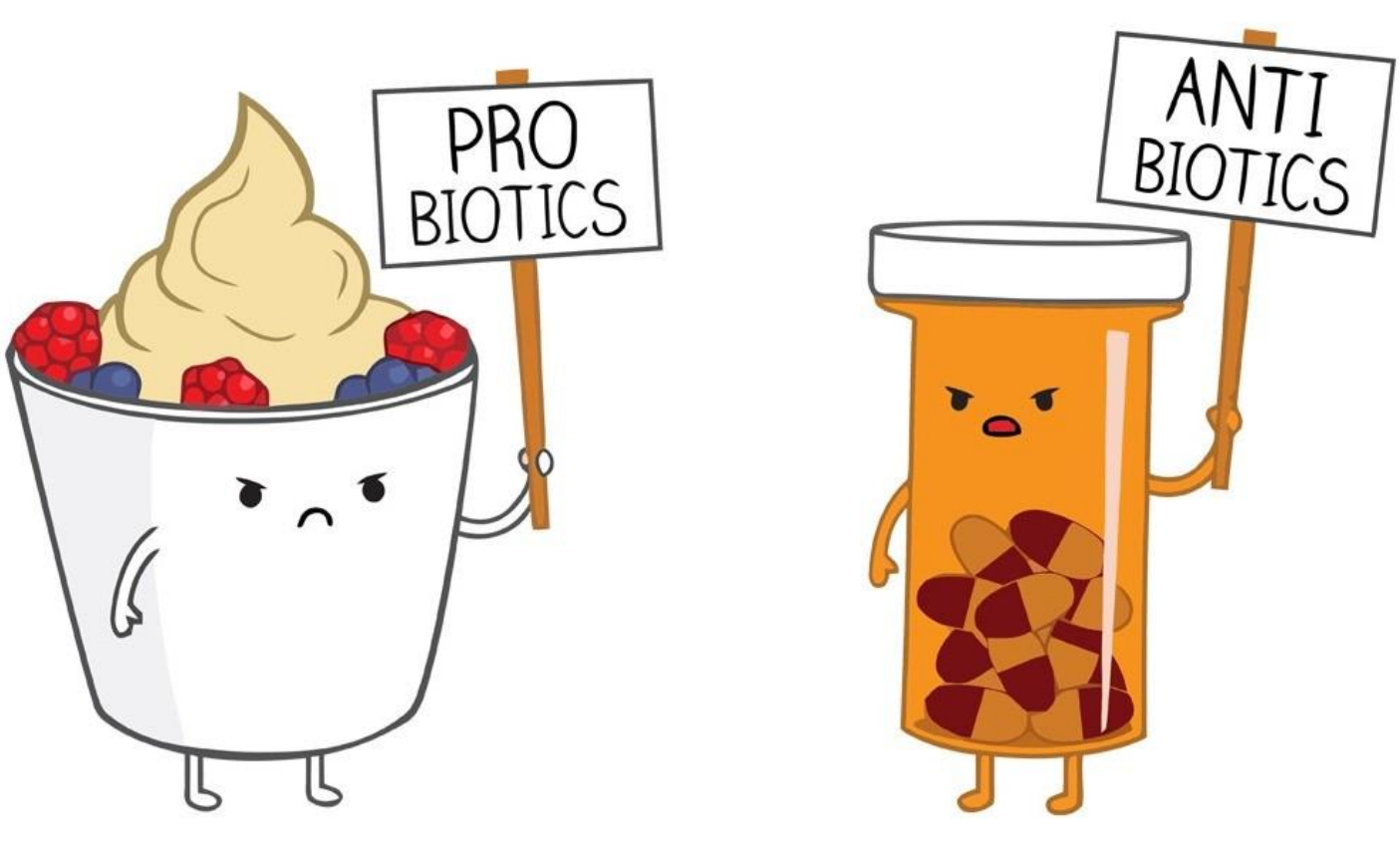
Figure 2. CAP-specific tailing activity of reverse transcriptase in the presence of manganese. SuperScript reverse transcriptase was tested in a primer extension reaction containing an *in vitro* transcribed RNA template with either 5′-capped (lanes 1–3) or -hydroxyl termini (lanes 4–6) using a radioactively labelled oligonucleotide primer. The reactions were performed as described in the text either in a standard reaction buffer (lanes 1 and 4) or supplemented with 1 mM (lanes 2 and 5) or 2 mM $MgCl_2$ (lanes 3 and 6). Reaction products were analysed on a denaturing 10% polyacrylamide/8 M urea gel and quantified using a PhosphorImager.

The CAP-dependent addition of three to five non-templated cytosine residues can be performed also after completion of cDNA synthesis using the standard reaction buffer in a post-incubation in the presence of manganese (+1, 10%; +2, 5%; +3, 57%; +4, 28%).

Mrakiealla aquatica Amylase

- Future plans
  - Confirm the sequence of the gene
  - Analysis *in silico:* signal sequence, hydrophobic profile and localization in cell, posttranslation modifications
  - Recombinant production

Thank you for your attention!



- MMLV reverse transcriptase – Moloney murine leukemia virus
  - able to add a few non/template nucleotides (mostl C) to the 3‘-end of a newly synthesized cDNA strand