



APAgene™ GOLD-RT Genome Walking Kits

Cat. # BT901-RT

Quantity 10 walks

Description The APAgene™ GOLD Genome Walking Kit can enable rapid isolation of unknown sequences which flank known gene sequences. Examples include:

- Genomic sequences flanking transgenes (including T-DNAs, gene traps, transposons), sequence-tagged sites (STSs) or expressed sequence tags (ESTs);
- Insert ends of large clones such as P1, YAC and BAC DNAs;
- 5' promoter control regions and 3' transcriptional terminators of cDNAs;
- cDNA walking, 5' and 3' RACE from first-strand cDNAs;
- Other localized sequences flanking known sequences from large clones and genomic DNAs.

PCR products can directly be used for sequencing, cloning or as probes.

Storage

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APAgene™ **GOLD** Genome Walking Kit (RT)

#BT901-RT (10 walks)

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1.0 Introduction

The APAgene™ GOLD Genome Walking Kit can enable rapid isolation of unknown sequences which flank known gene sequences. Examples include:

- Genomic sequences flanking transgenes (including T-DNAs, gene traps, transposons), sequence-tagged sites (STSs) or expressed sequence tags (ESTs);
- Insert ends of large clones such as P1, YAC and BAC DNAs;
- 5' promoter control regions and 3' transcriptional terminators of cDNAs;
- cDNA walking, 5' and 3' RACE from first-strand cDNAs;
- Other localized sequences flanking known sequences from large clones and genomic DNAs.

PCR products can directly be used for sequencing, cloning or as probes.

1.1 Amplification process

Our patented technology relies on a combination of our degenerate random tagging primers (DRT) and customer-provided gene-specific primers (GSP). Typically, three nested gene specific primers (GSPa, GSPb, and GSPc) are required in the reaction for the amplification of flanking sequences. DRT primers are universal binding primers consisting of 3 parts: 1) a *degenerate sequence*, 2) a *random sequence*, and 3) a *tagging sequence*. All the DRT primers share the same tagging sequence, but have different random sequences.

Three reactions are designed to amplify targeted sequences: a primary PCR, followed by two nested PCR.

1. The primary PCR is a 2-Step PCR that takes place in one tube. In Step 1, single strand DNA (ssDNA) fragments are produced by single primer extension reaction using a customized gene specific primer (GSPa). The reaction is repeated four times in four individual tubes. Step 2 immediately follows in which four different DRT primers, along with a 5X DRT binding mixture, are added individually to the four reaction tubes.
2. During the first nested PCR, a nested gene specific primer (GSPb) in combination with a long universal tagging primer (UAP-N1) are used for the four nested PCRs. UAP-N1 is a hairpin-containing primer, making non-specific amplification much less efficient compared to targeted sequences.
3. The second nested PCR use a nested gene specific primer (GSPc) in combination with a short universal tagging primer (UAP-N2) to increase yield of the specific product for subsequent sequencing or cloning.

The success of the walking reaction relies on the successful binding of the DRT primers to the flanking sequence during the primary PCR. Our patented DRT primers, along with our optimized cycling conditions and PCR buffer, greatly enhance the amplification process.

1.2 Kit components

Important (work on ice): Immediately upon receiving the kit add TE buffer (10mM Tris-HCl + 1mM EDTA pH8.0) to all the tubes (as indicated in Table A) to dissolve the reagents. Kit can then be stored at -20°C.

Table A: APAgene™ GOLD Genome Walking Kit components:

Components	TE buffer volume to be added:
APAgene™ 2X Premix	2 x 500µl
5X DRT binding mixture	200ul
DRT Primer A	10.0ul
DRT Primer B	10.0ul
DRT Primer C	10.0ul
DRT Primer D	10.0ul
UAP-N1 (20pmol/µl)	16.0ul
UAP-N2 (20pmol/µl)	16.0ul

APAgene™ 2X Premix contains a PCR buffer, dNTPs and the PCR annealing enhancer (to increase PCR specificity). The 5X DRT binding mixture contains a PCR buffer and the DRT binding enhancer. With the exception of the TE buffer, all the other reagents are in dried-pellet form. An inert red dye has been added to help visualize the pellet (will not have impact on PCR reactions).

Additional components that are required, but not included, are:

- Customer-designed gene-specific primers (GSPa, GSPb, GSPc)
- Template DNA
- Taq DNA polymerase
- Sterilized, Deionized H₂O
- 1kb DNA ladder marker

Important: APAgene™ GOLD Genome Walking Kit requires the use of high quality Taq DNA polymerases (NOT INCLUDED). Recommended Taq DNA polymerases are:

- Qiagen (Cat# 201203)
- Invitrogen (Cat# 10342)
- Clontech (Cat# 639108)

The sequences of the DRT primers are as follows. Please note that the letter X symbolizes degenerate and/or cleavable nucleotides.

DRT Primer	Sequences
DRT Primer A:	5'-GAACACGCGTCGTTTACCTCCXXXXGXXXXTAGT-3'
DRT Primer B:	5'-GAACACGCGTCGTTTACCTCCXXXXXTAGT-3'
DRT Primer C:	5'-GAACACGCGTCGTTTACCTCCXXXXXTCAT-3'
DRT Primer D:	5'-GAACACGCGTCGTTTACCTCCXXXXGXXXXCCTG-3'
UAP-N1:	5'- AGT CGG GAA GCA GTG GTA TCA ACG CAG AGT GGC CAT TAC GGC C GAA CAC GCG TCG TTT ACC T -3'
UAP-N2:	5'- C CUG GAA GCA GTG GTA TCA ACG-3'

2.0 Genomic Walking Protocol

2.1 Prior to beginning

Important: Thaw all reagents on ice. Should precipitation be observed in the APAgene™ 2X Premix tube, please place tube at room temperature until fully dissolved. All PCR preparations must be done on ice before loading in a pre-warmed PCR machine. Always pre-warm the PCR machine to 95°C before loading the samples in order to reduce non-specific amplification.

2.1.1 Preparing template DNA

DNA template (i.e. genomic DNA, BAC DNA or cDNA) should be highly pure, intact and free from contamination. Avoid a high content of salt, phenol or other DNAs. Please note that the amplification process is extremely sensitive to DNA impurities.

2.1.2. Designing gene-specific primers

Before performing the PCR, you must design gene-specific primers. The accuracy and quality of the gene specific primers are of great importance as they will influence the success of the walking reaction. The gene specific primers anneal to the known sequences, such as transgenes, STSs, ESTs, cDNAs and cloning vector.

Design GSPa to anneal about 150 bp from the end of the known sequence, GSPb can be designed to anneal immediately adjacent to GSPa. It's recommended that GSPc anneal about 70 bp from the end if product is to be used for direct sequencing as this will help verify the junction between the known and the resulted flanking sequences.

The following criteria should be considered for GSP design:

1. Verify the ends of the known sequence. This will determine which part of the known sequences will be used for GSP design.
 - Perform PCR to verify that the template DNA contains the ends of the known sequences (usually 150-200bp long). Occasionally, the transgene insertion may be truncated and will not contain the end sequences. Design primers and perform PCR to amplify the expected amplicons corresponding to the end sequences. If the template DNA does not contain the sequences for GSP design, redesign the primers further away from the end and amplify again.
 - If cDNA is the starting material, but genomic DNA or BAC DNA is used as a template, intron(s) may affect the amplification. GSPs used for walking should not overlap an exon/intron junction.
 - If the known sequence (cDNA or genomic DNA sequence) is obtained from one individual, but DNA extracted from another individual is used as template DNA in PCR walking, you should amplify the known sequences using the second DNA as a template and perform a double-strand sequencing of the amplified products. If there are base differences

between the first and the second sequence, use the second for designing GSPs.

2. Select appropriate direction (5' or 3').

- To locate transgenes, select the transgene border flanked by genomic DNA to design GSPs. This is very important to determine the genomic sequences flanking inverted repeats of transgenic insertions. If you can't determine which border you have to select, two sets of GSPs should be designed according to both end sequences and used in two separate reactions.

3. Some import criteria for GPS design.

- Primer sequences should have the following features: 23-28 bases long; 40-60% GC content; T_m of 66-75°C for GSPa and 60-70°C for GSPb and GSPc. Please see appendix for examples.
- Avoid primers with repeated sequences in the template.
- Avoid ambiguous bases in the primer sequence.
- Avoid internal secondary structures such as hairpins.
- Avoid strings of more than 3 identical bases at the 3' end and more than 7 in the middle of your primer sequence.

2.2 Primary PCR

2.2.1 Verifying the specificity of GSPa

Because APAGene™ GOLD Genome Walking Kit relies on the specific amplification of ssDNA with GSPa, verification of the specificity of GSPa is strongly recommended prior to the addition of the DRT primers.

Prepare one 15µl-reaction on ice according to Table 1. Mix gently and briefly spin down in a microcentrifuge. Place in thermocycler (pre-warmed to 95°C) and run Program 1 (Table 2).

Table 1: Verifying the specificity of GSPa

Components	Volume (µl)
H ₂ O	To 15µl
GSPa (20pmol/µl)	0.7
DNA template*	2.0
Taq DNA polymerase (5u/µl)	0.2 (not provided)
APAGene™ 2X Premix	7.5
Total	15

* Final concentration of the DNA template per 15 µl should be:
100ng to 1µg.

Table 2: Program 1

Step	Number of cycles	Temperature	Hold Time
1	1	94°C	4 minutes
2	25	94°C 65°C 67°C	30 seconds 30 seconds 4 minutes
3	1	68°C	10 minutes
4	1	4°C	Hold/End

Run a 10µl PCR mixture on an agarose gel. Check for non-specific amplification derived by GSPa. If one or more distinct bands appear, re-design primer because GSPa has produced a non-specific amplification.

If there are no distinct bands, proceed to the following step.

2.2.2 Step 1 of Primary PCR:

This step will amplify single-stranded targeted DNA fragments by linear amplification using GSPa.

Set up four (4) reactions in four (4) individual tubes on ice according to Table 3. Label tubes A1-D1. Mix gently and briefly spin down in a microcentrifuge. Place in thermocycler (pre-warmed to 95°C) and run Program 1 (Table 2). Once PCR is completed, place the tubes on ice and immediately proceed with Step 2 of the Primary PCR.

Important: Do not stop at this step, since single-strand DNA is not stable.

Table 3: Step 1 of Primary PCR

Components	Volume (μl)
H ₂ O	To 15μl
GSPa (20pmol/μl)	0.7
DNA template	1.0-2.0
Taq DNA polymerase (5u/μl)	0.2 (not provided)
APAgene™ 2X Premix	7.5
<i>Total</i>	15

2.2.3 Step 2 of Primary PCR

This step will anneal the DRT primers to the amplified ssDNA fragments, and then amplify dsDNA.

While working on ice, open the caps of tube A1-D1 carefully. Then, add 4.0μl of DRT binding mixture, 1.0μl of one of the four DRT primers, as well as 1 unit of Taq DNA polymerase to each of the four tubes as indicated in Table 4. Mix gently and briefly spin down in a microcentrifuge. Place in a thermocycler (pre-warmed to 95°C) and run Program 2 (Table 5).

Table 4: Step 2 of Primary PCR

Tube	Add:
A1	4.0μl of DRT binding mixture + 0.8μl of DRT primer A + 0.2μl of Taq DNA polymerase (5u/μl)
B1	4.0μl of DRT binding mixture + 0.8μl of DRT primer B + 0.2μl of Taq DNA polymerase (5u/μl)
C1	4.0μl of DRT binding mixture + 0.8μl of DRT primer C + 0.2μl of Taq DNA polymerase (5u/μl)
D1	4.0μl of DRT binding mixture + 0.8μl of DRT primer D + 0.2μl of Taq DNA polymerase (5u/μl)

Table 5: Program 2

Step	Number of cycles	Temperature	Hold Time
1	1	94°C	4 minutes
2	10	42°C 67°C	60 seconds 60 seconds
3	1	67°C	5 minutes
4	7	94°C 65°C 67°C 94°C 65°C 67°C 94°C 45°C 67°C	30 seconds 30 seconds 3 minutes 30 seconds 30 seconds 3 minutes 30 seconds 60 seconds 3 minutes
5	1	68°C	15 minutes
6	1	4°C	Hold/End

2.3 First nested PCR

Make a 50x Dilution of the PCR mixture from the primary PCR (Step 2.2.3) using ddH₂O. Store undiluted products at -20°C.

Label four tubes A2-D2. As described in Table 6, set up four 15µl-reactions on ice using four diluted PCR products as templates, respectively. Mix gently and briefly spin down in a microcentrifuge. Place in a thermocycler (pre-warmed to 95°C) and run Program 3 (Table 7).

Table 6: First nested PCR

Components	Volume (µl)
H ₂ O	To 15µl
GSPb (20pmol/µl)	0.4
UAP-N1	0.3
50x diluted DNA template	0.5
APAgene™ 2x Premix	7.5
Taq DNA polymerase (5u/µl)	0.2 (not provided)
<i>Total</i>	15

Table 7: Program 3

Step	Number of cycles	Temperature	Hold Time
1	1	94°C	4 minutes
2	35	94°C 62°C 67°C	30 seconds 30 seconds 3 minutes
3	1	68°C	10 minutes
4	1	4°C	Hold/End

2.4 Second nested PCR

Make a 50x dilution of the PCR mixture from the first nested PCR (Step 2.3) using ddH₂O. Store undiluted products at -20°C.

Label tubes A3-D3. As described in Table 8, set up four 15µl-reactions using four diluted PCR products as templates, respectively. Mix gently and briefly spin down in a microcentrifuge. Place in a thermocycler (pre-warmed to 95°C) and run Program 4 (Table 9).

Table 8: Second nested PCR

Components	Volume (µl)
H ₂ O	To 15µl
GSPc (20pmol/µl)	0.4
UAP-N2	0.3
50x diluted DNA template	0.5
APAgene 2x Premix	7.5
Taq DNA polymerase (5u/µl)	0.2 (not provided)
<i>Total</i>	15

Table 9: Program 4

Step	Number of cycles	Temperature	Hold Time
1	1	94°C	4 minutes
2	29-32*	94°C 60°C 67°C	30 seconds 30 seconds 3 minutes
3	1	68°C	10 minutes
4	1	4°C	Hold/End

**If signal is weak, cycle number may be increased up to a maximum of 32 cycles.*

At the end of the third PCR, analyze the PCR results by electrophoresis on a 1% agarose gel in 1x TAE buffer. In most cases, you will observe one or more ranging from 300-4,000bp (Please see appendix for examples). Excise the specific band and purify it using your method of choice. Purified products can then be cloned or directly sequenced using GSPc.

3.0 Troubleshooting Guide

If you cannot obtain a clear sequence by directly sequencing the PCR products, it is strongly recommended that the products be cloned prior to sequencing. Because of directional ligation, only specific sequences can be selectively cloned when using this kit. Furthermore, by using sticky-end ligation, it offers much higher cloning efficiency than blunt-end or TA-cloning.

In some cases, the third PCR produces multiple bands. There may be several explanations:

- i. There are multiple copies of the starting gene with different flanking sequences, such as contaminated DNA or transgenic DNA with multiple transgene copies in different genomic DNA loci. These fragments are amplified because DRT primers will bind to the different flanking sequences. In this case, noisy signals are observed when directly sequencing without cloning. All these fragments should be cloned and sequenced separately to identify the desired sequence.
- ii. If you are certain that there is only one copy of the starting gene, then multiple bands may result from multiple binding of the DRT primer to the same target area. These fragments are of same origin but varying in size. Clear sequences can be obtained by directly sequencing in most cases. However, cloning is required for difficult templates.
- iii. The GSPs have binded non-specifically and some fragments are non-specific. In this case, weak or noisy signals are observed when directly sequencing without prior cloning. Re-design your GSPs and re-start the reaction from the primary PCR.

4.0 Examples for GSP design and walking results

4.1 Examples of sequences and GSP primers

4.1.1 Sequence for human genome walking and GSP primers

5'AGAAACCCGACCTACCACGGCTTGCTCCTTCCCTTT**CCTCGCAGAGGTTTTCTCTCCAG**
CCCTGGACTCCTGTAGGATCTCAGCTC3'>>>walking direction

1. GSPa: 5'-**AAC CCG ACC TAC CAC GGC TTG CTC CTT**-3'
2. GSPb: 5'-**CCT CGC AGA GGT TTT CTC TCC AGC**-3'
3. GSPc: 5'-**GCC CTG GAC TCC TGT AGG ATC TCA**-3'

4.1.2 Sequence for mouse genome walking and GSP primers

5'AACGTCTGTGTCCGTGTGCATGTTGCATGTATGACAGAAAGGATAAAGCTACCAC**GGTAG**
ACATATTCTCAGGGCTGCTGGAGAAGAGAATGAAAAAGAGG**TTGTGGA**ACTGAGGCCACAT
TCTTT3'>>>walking direction

1. GSPa: 5'-**TGC ATG TTG CAT GTA TGA CAG AAA GG**-3'
2. GSPb: 5'-**GGT AGA CAT ATT CTC AGG GCT GCT GG**-3'
3. GSPc: 5'-**TTG TGG AAC TTG AGG CCA CAT TCT**-3'

4.2 Walking results

Using 100ng of genomic DNA as starting material in a linear amplification, Figure 1 shows the results of 2nd nested PCR. All fragments labeled with an arrow were confirmed to be specific by sequencing.

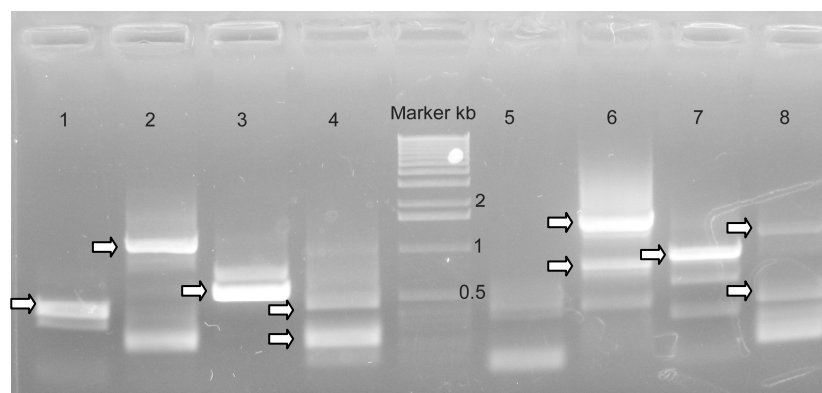


Figure 1: Gel results for two genome walkings (human and mouse) using four DRT walking primers: Lane 1: DRT B (Mouse), Lane 2: DRT B (Human), Lane 3: DRT D (Mouse), Lane 4: DRT D (Human), Lane 5: DRT A (Mouse), Lane 6: DRT A (Human), Lane 7: DRT C (Mouse), Lane 8: DRT C (Human). Arrowed fragments were cloned and sequenced.