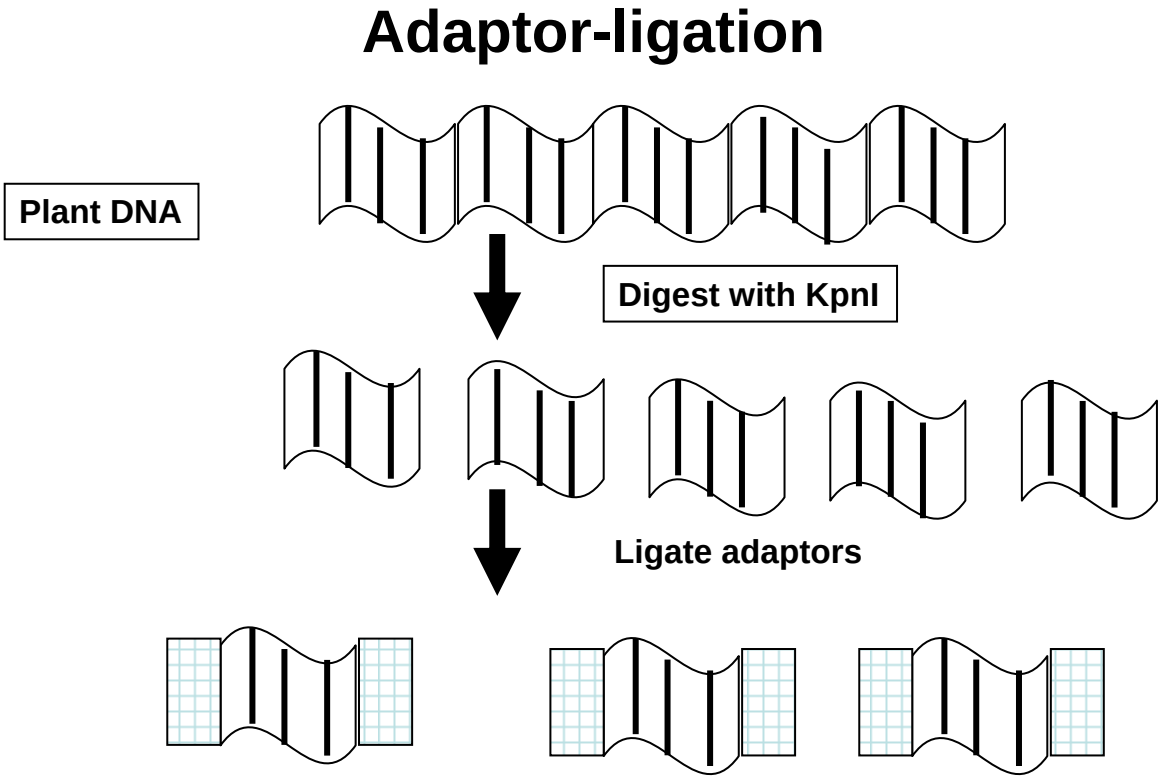
Annex 1

Adaptor-Primers

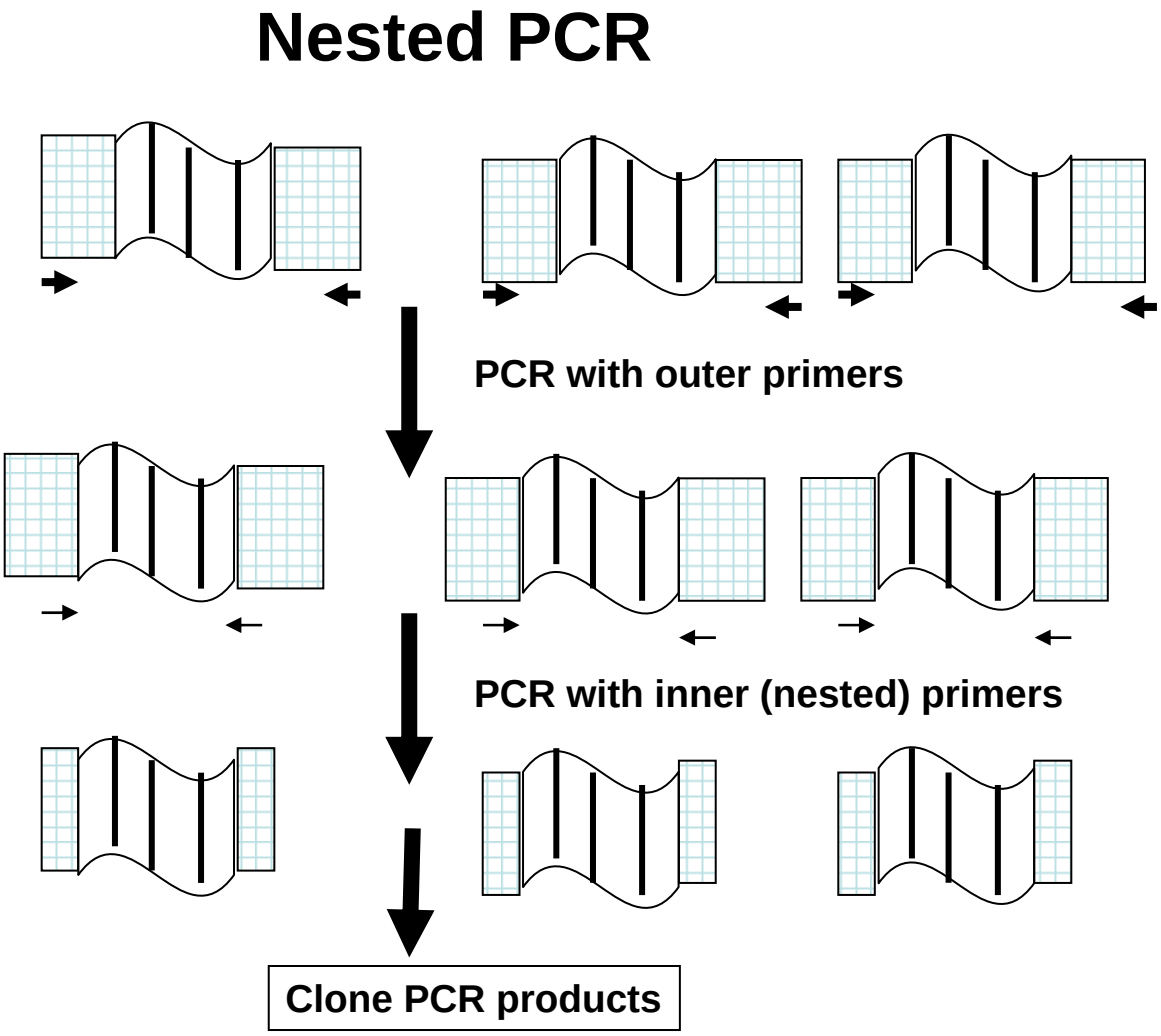
&

Nested PCR

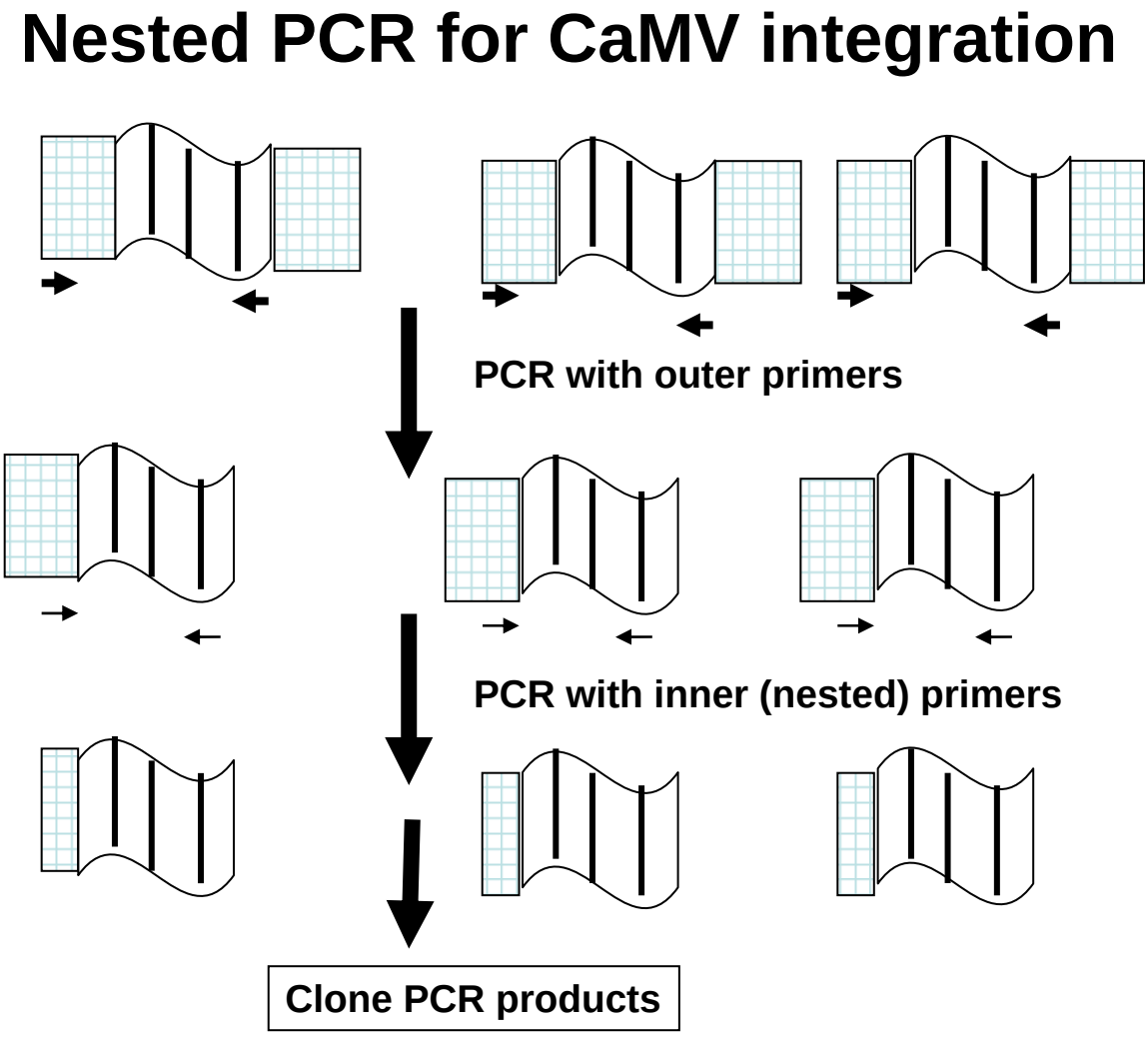
Nested PCR using adaptor-primers is a procedure where adaptor-linkers are first ligated onto restriction enzyme digested DNA. This creates known flanking sequences that can be amplified further by a nested PCR.



The first round of nested PCR uses other primers complementary to the adaptor-linkers or an adaptor-linker and an internal priming site. The second round uses primers internal to the first primers.



The same approach, but with different primers is used for examining integration of CaMV into the plant genome, but not for integration of CaMV into the 35S RNA promoter in a GM plant.



To detect integration into non-GM plants, the outer forward primer is to the adaptor-linker sequence and the outer reverse is to the integrated CaMV 35S RNA promoter sequence. The nested PCR then is done with an inner (nested) PCR primer to the adaptor linker sequence, but overlapping with the first (outer primer), and with an inner (nested) PCR primer to the 35S RNA promoter region. The nucleotide sequence and/or border positions of these primers are described below.

To detect integration into the 35S RNA promoter of GM plants, nested PCR was not required, but just PCR using a forward primer derived from CaMV gene VI and a reverse primer complementary to GFP sequences. The nucleotide sequence and/or border positions of these primers are described below.

Primers:

Detection of integration in non-GM plants.

Adaptor Sequence, top strand, bottom strand with amine block on 3' end.

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CCTAAGCTTCCGAATTCCAGTTACACGGCCGGTAC
            Amine group-TGCCGGC
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Sequence of plant DNA restriction fragment with a *Kpn*I site at the left terminus and CaMV 35S RNA promoter sequences present inside the genome. Primers were then used to the above adaptor-linker, and to the underlined CaMV sequences.

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    C.plant--GCTCCTACAAATGCCATCA---TGACGCACAATCCCACTATC--plant
CATGG.plant--CGAGGATGTTTACGGTAGT---ACTGCGTGTTAGGGTGATAG--plant
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Outer round PCR primers Forward (adaptor) and Reverse (CaMV) for 40 cycles. The product was diluted 1:49 with water and use in nested PCR. The nested PCR round primers were Forward (adaptor) and Reverse (CaMV), for 40 cycles. The PCR product was then cloned into pGemT-Easy (Promega).

Reverse (nt 7384-7365) and Nested Reverse (7208-7190) primers designed to 35S RNA promoter region of Cauliflower mosaic virus, complete genome NCBI accession NC_001497.

Detection of integration in GM 35S-GFP plants.

Forward primer designed to CaMV gene VI sequence, nt 5779-5801 from Cauliflower mosaic virus, complete genome, NCBI accession NC_001497.
Reverse primer designed to GFP sequence, nt 430-456 of NCBI accession AY292281.

Detection of GFP gene in transgenic plants (for Tasks 7, 9).

Forward primer. P5': CAC TGG AGT TGT CCC AAT TCT T. Homologous to nt 49-60 of the *GFP* gene.

Reverse primer. P3': CAT GAG CTC TTA TTT GTA TAG TTC ATC C.
Complementary to nt 725-742 of the *GFP* gene, and an added *Sac*I terminus.

Detection of CaMV insertion by PCR with primers to CaMV gene III and gene VI.

Gene III Forward primer nt 1831-1851; Gene III Reverse primer nt 2138-2158 of CaMV.

Gene VI Forward primer nt 5779-5801; Gene VI Reverse primer nt 6350-6370 of CaMV. [CaMV complete genome, source: NCBI accession NC_001497.]

PCR Conditions:

Detection of 35S RNA promoter (Fig. 4):

The PCR reactions were done by denaturation at 94 ºC for 3 min, followed by 40 cycles of inclubation at 94 ºC for 1 min, 54 ºC for 1 min, 72 ºC for 1min, and a final incubation at 72 ºC for 10 min. The two primers were both to the 35S RNA promoter region: the Forward primer was 5'-GCTCCTACAAATGCCATCA-3' (derived from nt 7190-7208) and the Reverse primer was 5'-GATAGTGGGATTGTGCGTCA-3' (complementary to nt 7383-7365), corresponding to the complement of Reverse primer and the same as the Nested Reverse primer described above, respectively.

Detection of integration into non GM plants:

The PCR reactions (both rounds) were done by denaturation at 94 ºC for 3 min, followed by 40 cycles of incubation at 94 ºC for 1 min, 55 ºC for 1 min, 68 ºC for 6.5 min, and a final incubation at 68 ºC for 10 min. The primers used are described above.

Detection of integration into GM plants:

The PCR reactions were done by denaturation at 94 ºC for 3 min, followed by 35 cycles of incubation at 94 ºC for 30 sec, 50 ºC for 30 sec, 72 ºC for 1.5 min, and a final incubation at 72 ºC for 10 min. The primers used were described above.

Dectection of integration of CaMV in transformed *A. thaliana* by detecting gene III and gene VI:

The PCR reactions were done by denaturation at 94 ºC for 3 min, followed by 40 cycles of incubation at 94 ºC for 1 min, 55 ºC for 1 min, 72 ºC for 1 min and a final incubation at 72 ºC for 10 min. The primers used were described above.