

# SMART: Atlas Switch Mechanism At the 5' end of Reverse Transcript amplification of RNA

## Outline

Amplification can be used to produce sufficient RNA for labelling when the amount of biological material is limiting. SMART = Atlas Switch Mechanism At the 5' end of Reverse Transcript method. Amplified DNA is then labeled using the Klenow labelling of double stranded DNA protocol.

## Equipment and reagents

- SMART II A chimeric primer: 5'-AAG-CAG-TGG-TAT-CAA-CGC-AGA-GTA-CGC-888-3'
  - ◆ 8 = riboG
- 3' SMART CDS Primer IIA:  
5'-AAG-CAG-TGG-TAT-CAA-CGC-AGA-GTA-CTT-TTT-TTT-TTT-TTT-TTT-TTT-TTT-TTT-VN-3'
  - ◆ V = G+A+C
  - ◆ N = A+C+G+T
- 5' PCR Primer II: 5'-AAG-CAG-TGG-TAT-CAA-CGC-AGA-GT-3'
- dATP, dCTP, dTTP and dGTP (Sigma; Cat. No. dNTP-100A)
- DEPC - Diethyl pyrocarbonate (Sigma; Cat. No. D 5758)
- DEPC-treated MilliQ water
- Superscript III Reverse Transcriptase (Invitrogen; Cat. No. 18080-044)
- Advantage II Polymerase Mix (Clontech; Cat. No. 639201)
- QIAquick PCR Purification Kit (Qiagen; Cat. No. 28104)
- Bioprime DNA Labeling System (Invitrogen; Cat. No. 18094-011)
- Cy3 dCTP (GE Healthcare Bio-Sciences AB; Cat. No. PA 53021)
- Cy5 dCTP (GE Healthcare Bio-Sciences AB; Cat. No. PA 55021)
- AutoSeq G-50 column (GE Healthcare Bio-Sciences AB, Cat. No. 27-5340-01)
- Sonicated Salmon Sperm DNA (Invitrogen; Cat. No. 15632-011)
- Hettich micro 20 centrifuge
- Grant QBT2 hot-block
- GeneVac miVac vacuum concentrator
- Dyad thermal cycler (PCR block)

## Removal of RNase

All materials should be autoclaved and only handled using gloves. Glassware should be baked at 180 °C overnight. Water and solutions should be treated with DEPC. The work area can be cleaned using RNase Zap to further limit the risk of RNase contamination. If at all possible, it is also a good precaution to use a separate set of pipettes for RNA work.

## Procedure

### RNA amplification

#### Making the 10 mM dNTP mix:

1. Make a large 10 mM dNTP mix for the RT-reaction and second strand synthesis
  - ◆ 100 µl 100mM dNTA
  - ◆ 100 µl 100mM dNTT

- ◆ 100 µl 100mM dNTG
  - ◆ 100 µl 100mM dNTC
2. Then make up to 1 ml using DEPC-water
  3. Aliquot the dNTP mix and then store at -20 °C

**Reverse Transcription reaction:**

The following steps are performed in 200 µl PCR tubes and the PCR block

1. Take up to 2.25 µL of the extracted RNA (small scale RNA extraction protocol) and add 0.75 µl DEPC-water
2. Add 1 µL 3'-SMART CDS Primer IIA (10 µM)
3. Add 1 µL SMART IIA chimeric oligo (10 µM)
4. Incubate at 70 °C for 5 min
5. Then snap freeze on ice
6. Make up a premix for the RT reaction:
  - ◆ 2 µl 5x First Strand Buffer (from Superscript Kit)
  - ◆ 0.5 µL 0.1M DTT (from Superscript Kit)
  - ◆ 0.5 µL 10 mM dNTP mix
  - ◆ 1 µl 200 U/µL Superscript III
7. Add 5 µL to each sample and mix (total 10 µl)
8. Incubate at 46 °C for 1.5 hours
9. Then snap freeze on ice (store samples at 4 °C or -20 °C)

**cDNA amplification - SMART cycle number evaluation:**

To determine the optimal number of cycles required for generating products in exponential phase, 2 µL of the PCR products from a parallel run are visualised at every second cycle from cycle 18 to 26 on 1% agarose gel using one reaction of each sample type. The following steps are performed in 200 µL PCR tubes and the PCR block

10. Use 5 µL of the first-strand reaction (include negative and positive controls)
11. Make up a premix for the PCR reaction:
  - ◆ 36 µL DEPC-water
  - ◆ 5 µL 10x Advantage II PCR buffer (from Advantage II Polymerase Mix Kit)
  - ◆ 1 µL 10 mM dNTP mix
  - ◆ 2 µL 20 µM 5' PCR Primer II
  - ◆ 1 µL 50x Advantage II Polymerase Mix
12. Add 45 µL to each sample
13. PCR program:
  - ◆ step 1: 95 °C - 1 min
  - ◆ step 2: 95 °C - 5 sec
  - ◆ step 3: 65 °C - 5 sec
  - ◆ step 4: 68 °C - 6 min
  - ◆ cycle from step 2 for 27 more times
  - ◆ step 5: 4 °C - forever
14. Take 2 µL out of the PCR reactions after cycles 16, 18, 20, 22, 24 and 26
15. Run PCR aliots on 1% agarose gel and select cycle number below saturation

**cDNA amplification - using optimal PCR cycles:**

The following steps are performed in 200 µL PCR tubes and the PCR block

16. Use 5 µL of the first-strand reaction (include negative and positive controls)
17. Make up a premix for the PCR reaction:

Making the 10 mM dNTP mix:

- ◆ 36  $\mu\text{L}$  DEPC-water
  - ◆ 5  $\mu\text{L}$  10x Advantage II PCR buffer (from Advantage II Polymerase Mix kit)
  - ◆ 1  $\mu\text{L}$  10 mM dNTP mix
  - ◆ 2  $\mu\text{L}$  20  $\mu\text{M}$  5' PCR Primer II
  - ◆ 1  $\mu\text{L}$  50x Advantage II Polymerase Mix
18. Add 45  $\mu\text{L}$  to each sample
19. PCR program:
- ◆ step 1: 95  $^{\circ}\text{C}$  - 1 min
  - ◆ step 2: 95  $^{\circ}\text{C}$  - 5 sec
  - ◆ step 3: 65  $^{\circ}\text{C}$  - 5 sec
  - ◆ step 4: 68  $^{\circ}\text{C}$  - 6 min
  - ◆ cycle from step 2 for the optimal number of cycles determined above
  - ◆ step 5: 4  $^{\circ}\text{C}$  - forever

#### **Purification of the PCR products using QIAquick PCR Purification Kit:**

The following steps are performed in 1.5 mL microfuge tubes. Make sure that ethanol has been added to Buffer PE.

20. Add 5 volumes (250  $\mu\text{L}$ ) of Buffer PB to 1 volume (40  $\mu\text{L}$ ) of the reaction and mix
21. Apply sample to QIAquick column and spin at 13,000 rpm for 1 minute. Discard flow-through and place column back into same collection tube
22. Wash column with 750  $\mu\text{L}$  Buffer PE, spin at 13,000 rpm for 1 minute. Discard flow-through and place column back into same collection tube
23. Centrifuge column at 13,000 rpm for 1 minute
24. Place column into a clean 1.5 mL microcentrifuge tube
25. Add 30  $\mu\text{L}$  water to the center of the membrane, incubate for 1 minute, then spin at 13,000 rpm for 1 minute
26. Reduce the volume of the sample to 12-15  $\mu\text{L}$  in the speed vac
27. Measure concentration on Nanodrop. Optional: 2  $\mu\text{L}$  of the purified DNA can be run on an 1% agarose gel

#### **Klenow labelling:**

The following steps are performed in 200  $\mu\text{L}$  PCR tubes and the PCR block

28. Take up to 1  $\mu\text{g}$  double stranded DNA and make up to a total volume of 25  $\mu\text{L}$  with DEPC-water
29. Add 20  $\mu\text{L}$  2.5x Random Primer Reaction Buffer (supplied in the Bioprime Labelling System Kit)
30. Incubate at 100  $^{\circ}\text{C}$  for 5 minutes
31. Snap freeze on ice
32. Mix together the following to make a master mix:
  - ◆ 1  $\mu\text{L}$  10 X low-C dNTP mix
  - ◆ 2  $\mu\text{L}$  Cy3 or Cy5 dCTP
  - ◆ 1  $\mu\text{L}$  40U/ $\mu\text{L}$  Klenow
33. Add 4  $\mu\text{L}$  to each sample and mix by pipetting up and down
34. Incubate at 37  $^{\circ}\text{C}$  for 2 to 3 hours
35. Stop the reaction by adding 5  $\mu\text{L}$  Stop Buffer (supplied in the Bioprime Labelling System Kit)
36. Combine the Cy3 and Cy5 pairs
37. Reduce the volume to ~60  $\mu\text{L}$  in a speed vac with medium heat

#### **Probe clean-up:**

It is important to separate the fluorescently-labelled probe from any unincorporated dye and nucleotides. AutoSeq G-50 columns are quick and easy to use. Microcon 30 columns (Millipore) or Qiaquick PCR purification columns (Qiagen) work equally well.

cDNA amplification - using optimal PCR cycles:

Purify probe using an AutoSeq G-50 column as follows:

38. Prepare the G50 columns (need 2 columns per combined sample):
  - ◆ Resuspend the resin in the G-50 column by vortexing gently
  - ◆ Loosen the cap a quarter turn and snap off the bottom closure
  - ◆ Place the column in a 1.5 mL tube
  - ◆ Pre-spin column at 5,000 rpm (2000 x g) for 1 min to remove the buffer
  - ◆ Remove the top cap and place column in a new 1.5 mL tube
39. Pipette half the sample to the G50 columns onto the centre of the angled surface of the compacted resin bed being careful not to disturb the resin. Do not allow any of the sample to flow around the sides of the bed.
40. Centrifuge for 1 minute at 5000 rpm
41. Place a second column into the same 1.5 mL microfuge tube and then add the second half of the sample. Spin for 1 minute and 5,000 rpm.
42. Reduce volume of sample to between 2 to 5  $\mu$ L by placing in a speed vac with medium heat
43. Add 2  $\mu$ L of sonicated salmon sperm DNA

The samples have now been labelled and combined together for hybridisation to a microarray with the blocking agent, sonicated salmon sperm DNA. This material should now be used immediately to prevent any decay. Please refer to the appropriate hybridisation protocol for the next steps.

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