

Ambion WT Expression Kit Protocol (Version 120411)

(This protocol is based on the Ambion protocol and includes modifications made by the Genomics Lab based on users' experience.)

Prepare the Poly-A RNA Controls

To include premixed controls from the Affymetrix® GeneChip® Poly-A RNA Control Kit (PN 900433 or equivalent), add the reagents to the total RNA samples. Follow the instructions below. Ambion strongly recommends the use of poly-A controls for all reactions that will be hybridized to GeneChip® arrays.

Designed specifically to provide exogenous positive controls to monitor the entire target labelling process, a set of poly-A RNA controls supplied by Affymetrix can be added to the RNA prior to the First Strand Synthesis step.

Each eukaryotic GeneChip® probe array contains probe sets for several *B. subtilis* genes that are absent in eukaryotic samples (lys, phe, thr, and dap). These poly-A RNA controls are in vitro synthesized, and the polyadenylated transcripts for the *B. subtilis* genes are premixed at staggered concentrations.

The concentrated Poly-A Control Stock can be diluted with the Poly-A Control Dil Buffer and spiked directly into RNA samples to achieve the final concentrations (referred to as a ratio of copy number) summarized in Table 1.

Table 1: Final concentrations of Poly-A RNA controls when added to total RNA samples

Poly-A RNA spike	Final concentration (ratio of copy number)
Lys	1:100,000
Phe	1:50,000
Thr	1:25,000
Dap	1:6,667

The Poly-A RNA Control Stock and Poly-A Control Dil Buffer are provided in the Affymetrix® GeneChip® Poly-A RNA Control Kit (PN 900433 or equivalent) to prepare the appropriate serial dilutions based on table 2. If using greater than 100ng input RNA please note that the serial dilution is different.

Table 2: Serial dilution of Poly-A RNA control stock

Total RNA input amount	Serial dilutions				Volume of 4 th dilution to add to total RNA
	1 st dilution	2 nd dilution	3 rd dilution	4 th dilution	
100ng	1:20	1:50	1:50	1:10	2µl

Recommendation: Avoid pipetting solutions less than 2 µL in volume to maintain precision and consistency when preparing the dilutions.

IMPORTANT! Use non-stick RNase-free microfuge tubes to prepare all of the dilutions (not included).

To prepare the Poly-A RNA dilutions for 100 ng of total RNA:

1. Add 2 µl of the Poly-A Control Stock to 38 µl of Poly-A Control Dil Buffer for the first dilution (1:20).
2. Mix thoroughly and spin down to collect the liquid at the bottom of the tube.
3. Add 2 µl of the first dilution to 98 µl of Poly-A Control Dil Buffer to prepare the second dilution (1:50).
4. Mix thoroughly and spin down to collect the liquid at the bottom of the tube.
5. Add 2 µl of the second dilution to 98 µl of Poly-A Control Dil Buffer to prepare the third dilution (1:50).
6. Mix thoroughly and spin down to collect the liquid at the bottom of the tube.
7. Add 2 µl of the third dilution to 18 µl of Poly-A Control Dil Buffer to prepare the fourth dilution (1:10).
8. Mix thoroughly and spin down to collect the liquid at the bottom of the tube.
9. Add 2 µl of this fourth dilution to 100 ng of total RNA.

Note: The first dilution of the Poly-A RNA controls can be stored up to 6 weeks in a non-frost-free freezer at – 20 °C and frozen/thawed up to eight times.

Important notes:

Prepare master mixes for each step of the procedure to save time, improve reproducibility and minimise pipetting error.

Before preparing the master mixes, inspect the buffer mixes for precipitates. If necessary, warm the buffer mix(es) at <55°C for 1 to 2 min, or until the precipitate is fully dissolved.

When preparing master mixes, thoroughly vortex each buffer mix before use.

Mix the enzymes by gently flicking the tubes a few times before use. Keep the buffer mixes at room temperature until needed to prevent re-precipitation,

Keep the enzyme mixes on ice at all times.

Note the shaking settings are for the Vortex genie 2. Different plate shakers will need different settings.

Step 1: Synthesise first stand cDNA

Things to note:

1. Programme the thermocycler in advance with the heated lid option.
2. At the end of this protocol, place the tubes on ice and proceed immediately to "First Cycle: Second Strand cDNA Synthesis".

Method:

1. Add 2µl of the forth Poly A dilution to 100ng of Total RNA (Total RNA volume should not exceed 3µl).
2. Add RNase free water up to a volume of 5µl.
3. Prepare a first strand master mix, then dispense 5µl into a reaction tube
4. At **room temperature**, prepare the first strand master mix in nuclease-free tube

Component	Volume (µl)	Volume 13x
First strand buffer mix	4	52
First strand enzyme mix	1	13
Total	5.0	65

- a. Mix thoroughly by gently vortexing. Centrifuge briefly. Proceed immediately to next step
 - b. Transfer 5µl of the First-Strand Master Mix to the supplied PCR tubes
5. Add 5µl of total RNA, mix thoroughly by gently vortexing/flicking, centrifuge briefly then proceed to next step.
 6. Place the reaction tubes in a thermocycler and run the following programme (with heated lid fixed at 50°C). Reaction volume = 10µl:
 - i. 25°C for 60 minutes
 - ii. 42°C for 60 minutes
 - iii. 4°C for at least 2 min in thermal cycler
 7. Spin briefly and place on ice for 2 min to cool the plastic.

Note transferring second strand master mix to hot plastics may significantly reduce cRNA yields. Holding the first strand cDNA synthesis reaction at 4°C for longer than 10 minutes may significantly reduce cRNA yields.

Step 2: Synthesise second stand cDNA

Things to note:

1. Programme the thermocycler in advance with the heated lid option disabled or at room temperature.
2. At the end of this protocol, place the tubes on ice and proceed immediately to "In vitro transcription cRNA synthesis".

Method:

1. Prepare a second strand master mix, then dispense 50µl to each sample
2. **On ice**, prepare the second strand master mix in nuclease-free tube

Component	Volume (µl)	Volume 13x
Nuclease-free water	32.5	422.5
Second strand buffer mix	12.5	162.5
Second strand enzyme mix	5	65
Total	50	650

- a. Mix thoroughly by gently vortexing. Centrifuge briefly. Proceed immediately to next step
3. Transfer 50µl of the Second-Strand Master Mix to each (10µl) first-strand synthesis cDNA sample (Total = 60µl).
 4. Mix thoroughly by gently vortexing/flicking, centrifuge briefly then proceed to next step.
 5. Place the reaction tubes in a thermocycler and run the following programme (**with heated lid disabled**):
 - i. 16°C for 60 minutes
 - ii. 65°C for 10 minutes
 - iii. 4°C for at least 2 min in thermal cycler

6. Spin briefly and place on ice

Step 3: Synthesise cRNA by In Vitro Transcription

Things to note:

1. Programme the thermocycler in advance with the heated lid option set at 50°C.
2. At the end of this protocol, place the tubes on ice and proceed immediately to "cRNA purification" or freeze immediately.

Method:

1. Prepare an IVT master mix, then dispense 30µl to each sample
 - a. **At room temperature**, prepare the IVT master mix in nuclease-free tube

Component	Volume (µl)	Volume 13x
IVT buffer mix	24	312
IVT enzyme mix	6	78
Total	30	390

- b. Mix thoroughly by gently vortexing/flicking. Centrifuge briefly. Proceed immediately to next step
2. Transfer 30µl of the IVT master mix to each (60µl) second-strand synthesis cDNA sample (Total=90µl).
 3. Mix thoroughly by gently vortexing/flicking, centrifuge briefly then proceed to next step.
 4. Place the reaction tubes in a thermocycler and run the following programme (with heated lid at 50°C):
 - i. 40°C for 16 hours
 - ii. 4°C hold
 5. Spin briefly and place on ice or freeze immediately.

Step 4: cRNA purification

Things to note:

1. Preheat the bottle of elution solution to 50 to 58°C for at least 10 min
2. Add 100% ethanol to the bottle of Nucleic acid wash solution concentrate **before use**
3. Make sure the nucleic acid binding buffer concentrate is completely dissolved. If not, warm the solution to <50°C until the concentrate is solubilised.
4. Vortex the nucleic acid binding beads vigorously before use to ensure they are fully dispersed.
5. Use isopropanol at this step. Do not use ethanol

Method:

1. Prepare the cRNA binding mix:

Component	Volume (µl)	Volume 13x
Nucleic acid binding beads	10	130
Nucleic acid binding buffer concentrate	50	650
Total	60	780

1. Transfer each sample to a well of a U-bottom plate.
2. Add 60µl of cRNA binding mix to each sample. Pipette up/down 3 times to mix.
3. Add 60µl of **100% isopropanol** to each sample. Pipette up/down 3 times to mix
4. Seal plate with PCR film. Incubate for 5 minutes without shaking.
5. Shake gently for 2 min to thoroughly mix (setting 0 on Vortex genie 2 with plate adapter). The cRNA binds to the beads during this stage. Briefly centrifuge (<5 secs) to remove liquid from the film prior to its removal.

6. Capture the nucleic acid binding beads and discard the supernatant
 - a. Move the plate to a magnetic stand to capture the magnetic beads (~5min)
 - b. When capture is complete the mixture is transparent and the beads pellet on the sides of the wells.
 - c. Carefully aspirate and discard the supernatant without disturbing the magnetic beads, then remove the plate from the magnetic stand.
7. Wash twice with 100µl of nucleic acid wash solution
 - a. Add 100µl of nucleic acid wash solution to each sample, seal with PCR film then shake at moderate speed for 1 min (setting 1 on Vortex genie 2 with plate adapter). Briefly centrifuge (<5 secs) to remove liquid from the film prior to its removal.
 - i. Note the beads may not fully disperse at this stage but this does not affect yield.
 - b. Move the plate to a magnetic stand and capture the nucleic acid binding beads as in step 7.
 - c. Carefully aspirate and discard the supernatant without disturbing the nucleic acid binding beads, then remove the plate from the magnetic stand.
 - d. Repeat steps 8a to 8c to wash each sample again with 100µl of nucleic acid wash solution
 - e. Move the plate to a shaker and shake the plate vigorously for 1 min (setting 2 on Vortex genie 2 with plate adapter) to evaporate residual ethanol. Dry the solution until no liquid is visible, yet the pellet appears shiny. Additional time may be required. Do not over-dry the beads.
8. Elute cRNA with 40µl of preheated elution solution
 - a. Add to each sample 40µl of preheated (50 to 58°C) elution solution. Incubate without shaking for 5 min.
 - b. Seal the plate with PCR film vigorously shake the plate for 3 min (setting 2 on Vortex genie 2 with plate adapter). Check to make sure that the nucleic acid binding beads are fully dispersed. If not, continue shaking until the beads are dispersed and/or pipette up/down 3 times. Briefly centrifuge (<5 secs) to remove liquid from the film prior to its removal.
 - c. Move the plate to a magnetic stand to capture the nucleic acid binding beads
 - d. Transfer the supernatant, which contains the eluted cRNA, to a nuclease free PCR tube.
9. Place the cRNA on ice briefly or freeze immediately.

Assess cRNA yield and size distribution

Measure the absorbance at 260nm and 280nm of the eluate. Ensure the readings are in the linear range for the spectrophotometer. A ratio of the absorbances at 260 and 280nm should be around 2.0, with an acceptable range being 1.9 to 2.1. The first cycle cRNA

yield (concentration x eluate volume) should be > 20µg (Ambion quoted range 15 to 50µg depending upon tissue type). However, this may vary depending on tissue type. A lower yield indicates there may be a problem with the reaction.

A minimum of 455ng/µl is needed for the next step. This is equal to 10µg in a volume of 22µl.

Step 5: Synthesise 2nd cycle cDNA

Method:

1. On ice, prepare 455ng/µl cRNA. This is equal to 10µg cRNA in a volume of 22µl, if necessary; use nuclease free water to bring the cRNA sample to 22µl.
2. Combine 10µg cRNA and the random primers
 - a. On ice using supplied PCR tubes or plates, combine
 - i. 22µl of cRNA (10µg)
 - ii. 2µl of random primers
 - b. Mix thoroughly by gently vortexing/flicking. Centrifuge briefly. Place on ice.
3. Place the reaction tubes in a thermocycler and run the following programme (with heated lid at 75°C):
 - i. 70°C for 5min
 - ii. 25°C for 5min
 - iii. 4°C for 2 min
4. Spin briefly and place on ice.
5. Prepare the 2nd-cycle master mix on ice, and then add 16µl to each sample.
 - a. **At room temperature**, prepare the 2nd-cycle master mix in a nuclease free tube

Component	Volume (µl)	Volume 13x
2 nd -cycle buffer mix	8	104
2 nd -cycle enzyme mix	8	104
Total	16	208

- c. Mix thoroughly by gently vortexing. Centrifuge briefly. Proceed immediately to next step
6. Transfer 16µl of the 2nd-cycle master mix to each (24µl) cRNA/random primer sample.
7. Mix thoroughly by gently vortexing/flicking, centrifuge briefly then proceed to next step.
8. Place the reaction tubes in a thermocycler and run the following programme (with heated lid at 75°C (Reaction volume =40µl)
 - i. 25°C for 10 min
 - ii. 42°C for 90 min
 - iii. 70°C for 10 min
 - iv. 4°C for at least 2 min
9. Spin briefly and place on ice

Step 6: Hydrolyse using RNase H

Method:

1. Add 2µl of RNase H to the 2nd-cycle cDNA on ice
 - a. Mix by pipetting up and down 3 times to ensure all of the enzyme is dispensed from the pipette tip.
2. Mix thoroughly by gently vortexing/flicking. Centrifuge briefly
3. Place the reaction tubes in a thermocycler and run the following programme (with heated lid at 75°C):
 - i. 37°C for 45min
 - ii. 95°C for 5min
 - iii. 4°C for 2 min
4. Spin briefly and place on ice. Samples can be frozen at this point overnight at -20°C.

Step 7: 2nd-cycle cDNA purification

Things to note:

1. Preheat the bottle of elution solution to 50 to 58°C for at least 10 min
2. Add 100% ethanol to the bottle of Nucleic acid wash solution concentrate **before use**
3. Make sure the nucleic acid binding buffer concentrate is completely dissolved. If not, warm the solution to <50°C until the concentrate is solubilised.
4. Vortex the nucleic acid binding beads vigorously before use to ensure they are fully dispersed.
5. Note that **100% ethanol** is used in this step not isopropanol.

Method:

1. Prepare the cDNA binding mix:

Component	Volume (µl)	Volume 13x
Nucleic acid binding beads	10	130
Nucleic acid binding buffer concentrate	50	650
Total	60	780

2. Add 18µl of nuclease free water and 60µl of cDNA binding mix to each sample. Pipette up/down 3 times to mix. Transfer each sample to a well of a U-bottom plate.
3. Add 120µl of ethanol to each sample and pipette up/down 3 times to mix. Seal plate with PCR film.
 - a. Incubate for 5 minutes without shaking
 - b. Shake gently for 2 min to thoroughly mix (setting 0 on Vortex genie 2 with plate adapter). Briefly centrifuge (<5 secs) to remove liquid from the film prior to its removal.
4. Capture the nucleic acid binding beads and discard the supernatant
 - c. Move the plate to a magnetic stand to capture the magnetic beads
 - d. Carefully aspirate and discard the supernatant without disturbing the magnetic beads, then remove the plate from the magnetic stand.
5. Wash twice with 100µl of nucleic acid wash solution
 - a. Add 100µl of nucleic acid wash solution to each sample, seal the plate with PCR film then shake at moderate speed for 1 min (setting 1 on Vortex genie 2 with plate adapter). Briefly centrifuge (<5 secs) to remove liquid from the film prior to its removal.
 - b. Move the plate to a magnetic stand and capture the nucleic acid binding beads
 - c. Carefully aspirate and discard the supernatant without disturbing the nucleic acid binding beads, then remove the plate from the magnetic stand.
 - d. Repeat steps 5a to 5c to wash each sample again with 100µl of nucleic acid wash solution
 - e. Move the plate to a shaker and shake the plate vigorously for 1 min to evaporate residual ethanol from the beads (setting 2 Vortex genie 2 with plate adapter). Dry the solution until no liquid is visible, yet the pellet appears shiny. Additional time may be required. Do not over-dry the beads.
6. Elute cDNA with 30µl of preheated elution solution
 - a. Add to each sample 30µl of preheated (50 to 58°C) elution solution. Incubate without shaking for 5 min. Seal the plate with PCR film.
 - b. Vigorously shake the plate for 3 min (setting 2 on Vortex genie 2 with plate adapter), then check to make sure that the nucleic acid binding beads are fully dispersed. If not, continue shaking until the beads are dispersed and/or pipette up/down 3 times. Briefly centrifuge (<5 secs) to remove liquid from the film prior to its removal.
 - c. Move the plate to a magnetic stand to capture the nucleic acid binding beads.
 - d. Transfer the supernatant, which contains the eluted cDNA, to a nuclease free PCR tube.
7. Place the cDNA on ice briefly or freeze immediately overnight at -20°C.

Step 8: Assess cDNA yield and size distribution

Determine the concentration of cDNA solution by using 1.5µl of cDNA sample on the NanoDrop. Ambion suggests that using 10µg of input cRNA yields 6 to 9µg of cDNA.

Proceed to fragmentation and labelling of the single stranded cDNA.

Prepare a 1.5µl aliquot of each sample for size analysis using the Agilent 2100 Bioanalyser. Place tubes on dry ice to freeze and then store at -20°C, or proceed directly to “Fragmentation of single stranded cDNA”.

STEP 9: 100ng Whole Transcript Labelling Assay
Fragmentation of Single Stranded cDNA
Version 2, 14/02/2008

Things to note:

1. This protocol uses the GeneChip WT Terminal Labelling Kit, Affymetrix 900671 (30rxns) or 900670 (10rxns). Do not use the GeneChip WT Double-stranded WT Terminal Labelling Kit, as this is designed for another assay.
2. Programme the thermocycler in advance with the heated lid option.

Method:

1. Transfer single stranded cDNA (5.5µg) from the previous step to a 0.2ml RNase free tube.
2. Add RNase free dH₂O to make a total volume of 31.2µl.
3. Prepare a master mix. For each reaction add:

Component	Volume (µl)	Volume 13x
RNase free H ₂ O	10.0	130

10X cDNA Fragmentation Buffer	4.8	62.4
(10U/BI) UDG	1.0	13
(1,000U/BI) APE	1.0	13
Total	16.8	218.4

4. Mix by pipetting, spin briefly if necessary.
5. Transfer 16.8µl of the master mix to each of the reaction tubes. Mix by pipetting and spin briefly if necessary.
6. Place the tubes in a thermocycler and run the following programme (with heated lid):
 - i. 37°C for 60 minutes
 - ii. 93°C for 2 minutes
 - iii. 4°C for 2 minutes
 - iv. 4°C hold
7. Spin the tubes briefly.
8. Prepare a 1.5µl aliquot of each fragmented cDNA sample for size analysis using the Agilent 2100 Bioanalyser. Fragment size should be ~40-70bp. 1µl of the unfragmented samples (prepared previously) should be run along-side the fragmented samples for comparison. We recommend that you run all of your fragmented samples on the Bioanalyser.
9. Proceed immediately to "Labelling of Fragmented Single-Stranded DNA".

STEP 10: 100ng Whole Transcript Labelling Assay

Labelling of Fragmented Single Stranded cDNA

Version 2, 14/02/2008

Things to note:

1. This protocol uses the GeneChip WT Terminal Labelling Kit, Affymetrix 900671 (30rxns) or 900670 (10rxns). Do not use the GeneChip Double-stranded WT Terminal Labelling Kit, as this is designed for another assay.
2. Programme the thermocycler in advance with the heated lid option.

Method:

1. Transfer 45µl of the fragmented cDNA to a new 0.2ml RNase free tube.
2. Prepare a master mix. For each reaction add:

Component	Volume (µl)	Volume 13x
5X TdT Buffer	12.0	156
TdT	2.0	26

(5 mM) DNA Labelling Reagent	1.0	13
Total	15.0	195

3. Mix by pipetting, spin briefly if necessary.
4. Transfer 15µl of the master mix to each of the reaction tubes. Mix by pipetting and spin briefly if necessary.
5. Place the tubes in a thermocycler and run the following programme (with heated lid):
 - i. 37°C for 60 minutes
 - ii. 70°C for 10 minutes
 - iii. 4°C for 2 minutes
 - iv. 4°C hold
6. Spin the tubes briefly.
7. Using a pipette, measure the volume of labelled extract. For each sample, add sufficient RNase free water so that the final volume of the labelled extract is 60µl.
8. Freeze by placing tubes on dry ice and store at -80°C.

Bring the single-stranded cDNA to the Microarray Centre on dry ice (after prior consultation with a [Research Assistant](#) to arrange a convenient time). Note that each sample should be clearly labelled on both the top and side of the tube with a unique identifier that can be matched to the information provided in the online annotation tool.