Illumina RNA-Seq Protocol

Edited & Modified by:

Brandon Le, Anhthu Bui
and Kelli Henry

Written: April 2010

Revised: October 2011

This Protocol is adapted from the mRNA Sequencing Sample Preparation Guide

Illumina
Cat # RS-930-1001
Part # 1004898 Rev. D
September 2009

This protocol is used to convert total RNA into a library template for high-throughput sequencing using the Illumina Genome Analyzer (GA) system. This protocol should accompany the Illumina Sample Preparation Guide for all required kits and reagents. I've also included quick steps for the Qiagen purification kits. We've modified the protocol (especially the elution volumes) so that we can have a checkpoint at each step of the protocol.

Workflow:

Day 1	Day 2
1-10 μg Total RNA	Repair Ends
Purify and Fragment mRNA	Add 'A' Bases to 3' Ends
First Strand cDNA Synthesis	Ligate Adapters
Second Strand cDNA Synthesis	Purify Ligation Product
	PCR Amplification

Samples:

Purpose/Goals:

Table of Content

I. Purification of PolyA+ mRNA ***3***

A. Using the Magnetic Stand 3

B. Purify the mRNA 3

II. Fragment the mRNA ***3***

III. Synthesize the First Strand cDNA ***3***

IV. Synthesize the Second Strand cDNA ***3***

A. Make second strand cDNA 3

B. Purify the cDNA using the Qiagen Purification Kit 3

V. Perform End Repair ***3***

A. End Repair 3

B. Purify the cDNA using the Qiagen Purification Kit 3

VI. Adenylate 3' Ends ***3***

A. 3'End Adenylation 3

B. Purify cDNA Using the Qiagen MinElute PCR Purification Kit 3

VII. Ligate the Adapters ***3***

A. Adapter Ligation 3

B. Purify cDNA Using the Qiagen MinElute PCR Purification Kit 3

VIII. Purify the cDNA Templates ***3***

A. Run Samples on a 2% Gel 3

B. Purify cDNA Using the Qiagen QIAquick Gel Extraction Kit 3

IX. Enrich the Purified cDNA Templates ***3***

A. Amplify the adapter-ligated cDNAs 3

B. Purify cDNA Using the Qiagen PCR Purification Kit 3

X. Aliquot the Library

Appendix: Illumina Primers ***3***

Appendix: Using the Bio-Rad Experion System ***3***

Appendix: Ribogreen Assay Using Nanodrop ND-3300 Fluorospectrometer ***4***

I. Purification of PolyA+ mRNA

This process purifies the poly-A containing mRNA molecules using poly-T oligo-attached magnetic beads.

A. Using the Magnetic Stand

Illumina-Supplied
Sera-Mag Magnetic Oligo(dT) Beads
Bead Binding Buffer
Bead Washing Buffer
Ultra Pure Water
10 mM Tris Buffer

Using the Magnetic Stand
Follow these guidelines throughout the sample preparation protocol to prevent the beads from drying out. ***Allow sufficient time for all of the beads to be captured by the magnetic stand, not just until a pellet is visible.***

1. Place the tube containing the beads on the magnetic stand for at least 1–2 minutes to separate the beads and the buffer. ***Allow all of the beads long enough to be captured by the magnetic stand, not just until you see a pellet.***

2. Exchange the buffer using a pipette while the tube is on the magnetic stand.

3. Resuspend the beads thoroughly by vortexing with 0.5-1 second pulses. ***It is critical that the beads are thoroughly resuspended in the solution.***

4. Centrifuge the samples in a benchtop microcentrifuge for 1-2 seconds to remove any beads or liquid from the walls of the tube.

5. Repeat steps 1 through 4 as required.

Note: ***WE DID NOT USE THIS PROCEDURE TO OBTAIN POLYA+ mRNA. WE USED THE DYNABEAD SYSTEM INSTEAD.***

B. Purify the mRNA

1. Preheat one heat block to 65°C and the other heat block to 80°C.

2. Dilute the total RNA with nuclease-free water to 50 µl in a 1.5 ml RNase-free non-sticky tube.

3. Heat the sample in a preheated heat block at 65°C for 5 minutes to disrupt the secondary structures and then place the tube on ice.

4. Aliquot 15 µl of Sera-Mag oligo(dT) beads into a 1.5 ml RNase-free non- sticky tube.

5. Wash the beads two times with 100 µl of Bead Binding Buffer and remove the supernatant.

6. Resuspend the beads in 50 µl of Bead Binding Buffer and add the 50 µl of total RNA sample from step 3.

7. Rotate the tube from step 6 at room temperature for 5 minutes and remove the supernatant.

8. While the tube is incubating, aliquot 50 µl of Binding Buffer to a fresh 1.5 ml RNase-free non-sticky tube.

9. After the 5 minutes incubation, wash the beads from step 7 twice with 200 µl of Washing Buffer and remove the supernatant.

10. Add 50 µl of 10 mM Tris-HCl to the beads and then heat in the preheated heat block at 80°C for 2 minutes to elute the mRNA from the beads.

11. Immediately put the tube on the magnet stand, transfer the supernatant (mRNA) to the tube from step 8. Do not discard the used beads.

12. Place the samples aside and wash the beads twice with 200 µl of Washing Buffer.

13. Heat the samples in the preheated heat block at 65°C for 5 minutes to disrupt the secondary structures and then place the samples on ice.

14. Add the iced 100 µl of the mRNA sample from step 13 to the washed beads and rotate it at room temperature for 5 minutes, then remove the supernatant.

15. Wash the beads twice with 200 µl of Washing Buffer and remove the supernatant.

16. Add 17 µl of 10 mM Tris-HCl to the beads and heat in the preheated heat block at 80°C for 2 minutes to elute the mRNA from the beads.

17. Immediately put the tube on the magnet stand and then transfer the supernatant (mRNA) to a fresh 200 µl thin-wall PCR tube. The resulting amount of mRNA should be approximately 16 µl.

II. Fragment the mRNA

This process fragments the mRNA into small pieces using divalent cations under elevated temperature.

Illumina-Supplied	**User-Supplied**
5X Fragmentation Buffer	3 M NaOAC, pH 5.2
Fragmentation Stop Solution	70% EtOH
Glycogen	100% EtOH
Ultra Pure Water	

Procedure

1. Preheat a PCR thermal cycler to 94°C.
2. Prepare the following reaction mix in a 200 µl thin wall PCR tube (Total volume should be 20 µl): ***Note: you can start here with 100 ng of polyA+ RNA.***

   5X Fragmentation Buffer - 4 µl
   mRNA - 16 µl

3. Incubate the tube in a preheated PCR thermal cycler at 94°C for exactly 5 minutes.
4. Add 2 µl of Fragmentation Stop Solution.
5. Place the tube on ice.
6. Transfer the solution to a 1.5 ml RNase-free non-sticky tube.
7. Add the following to the tube and incubate at -80°C for 30 minutes or overnight as desired:

   3 M NaOAC, pH 5.2 - 2 µl
   Glycogen - 2 µl
   100% EtOH - 60 µl

8. Centrifuge the tube at 14,000 rpm (20,200 relative centrifugal force) for 25 minutes at 4°C in a microcentrifuge.
9. Carefully pipette off the EtOH without dislodging the RNA pellet.
10. Without disturbing the pellet, wash the pellet with 300 µl of 70% EtOH.
11. Centrifuge the pellet and carefully pipette out the 70% EtOH.
12. Air dry the pellet for 10 minutes at room temperature.
13. Re-suspend the RNA in **13 µl** of RNase-free water. ***Note: Save 1 µl for* Fluorescent Nanodrop reading *and 0.9 µl to run on the Experion HighSens RNA chip.***

Samples	Vol	Conc [ng/µl]	Total

Notes:

Note: We tested both 2.5' and 5' incubation time. *The 2.5' incubation time yield fragmented RNA sizes of (70 - 800 bases) as compared to the 5' incubation time (70 - 500 bases). Both time points, however, produced RNA fragments with two predominant sizes (~120 and 160 bases).*

Note: For steps 9 and 11, we saved the supernatant and labeled the samples as A- (just in case we accidentally pipet out the pellet).

Note: In step 12, you should see a white pellet.

III. Synthesize the First Strand cDNA

This process reverse transcribes the cleaved RNA fragments into first strand cDNA using reverse transcriptase and random primers.

Illumina-Supplied	**User-Supplied**
25 mM dNTP Mix	SuperScript II
Random Primers	100 mM DTT*
RNase Inhibitor	5X First Strand Buffer*
	***included with SuperScript II**

Procedure

1. Assemble the following reaction in a 200 µl thin wall PCR tube (Total volume should be 12.1 µl):

   Random Primers - 1.0 µl
   mRNA - 11.1 µl

2. Incubate the sample in a PCR thermal cycler at 65°C for 5 minutes, and then place the tube on ice.

3. Set the PCR thermal cycler to 25°C.

4. Mix the following reagents in the order listed in a separate tube. Multiply each volume by the number of samples being prepared. Prepare 10% extra reagent mix if you are preparing multiple samples (The total volume should be 6.9 µl).

   5X First Strand Buffer - 4.0 µl
   100 mM DTT - 2.0 µl
   25 mM dNTP Mix - 0.4 µl
   RNase Inhibitor - 0.5 µl

5. Add 6.9 µl of mixture to the PCR tube and mix well.

6. Heat the sample in the preheated PCR thermal cycler at 25°C for 2 minutes.

7. Add 1 µl SuperScript II to the sample and incubate the sample in a thermal cycler with following program:

   25°C for 10 minutes
   42°C for 50 minutes
   70°C for 15 minutes
   Hold at 4°C

8. Place the tube on ice.

Note: Program name on the BioRad Mycycler PCR machine: **RNASeq 1st strand**

IV. Synthesize the Second Strand cDNA

This process removes the RNA template and synthesizes a replacement strand generating double-stranded cDNA.

A. Make second strand cDNA

Illumina-Supplied	**User-Supplied**
Ultra Pure Water	QIAquick PCR Purification Kit
GEX Second Strand Buffer	(QIAGEN, part # 28104)
25 mM dNTP Mix	
RNaseH	
DNA Pol I	

Procedure

1. Preheat a PCR thermal cycler to 16°C.

2. Add 62.8 μl of ultra pure water to the first strand cDNA synthesis mix.

3. Add the following reagents to the mix:

GEX Second Strand Buffer	-	10.0 μl
25 mM dNTP Mix	-	1.2 μl

4. Mix well and incubate on ice for 5 minutes or until well-chilled.

5. Add the following reagents:

RNaseH	-	1 μl
DNA PolI	-	5 μl

6. Mix well and incubate at 16°C in a thermal cycler for 2.5 hours.

7. Follow the instructions in the QIAquick PCR Purification Kit to purify the sample and elute in 51 μl of QIAGEN EB buffer. (See **section IVb**).

8. At this point, the sample is in the form of double-stranded DNA.

9. It is OK to store the ds-cDNA at 4°C overnight (O/N).

Note: Program name on the BioRad Mycycler PCR machine: **RNASeq 2nd strand**

B. Purify the cDNA using the Qiagen Purification Kit

This protocol is designed to purify single- or double-stranded DNA fragments from PCR and other enzymatic reactions. Fragments ranging from 100 bp to 10 kb are purified from primers, nucleotides, polymerases, and salts using QIAquick spin columns in a microcentrifuge.

Important points before starting

1. Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).

2. All centrifugation steps are carried out at 17,900 x g (13,000 rpm) in a conventional tabletop microcentrifuge at room temperature.

3. Add 1 : 250 volume pH indicator I to Buffer PB (i.e., add 120 µl pH indicator I to 30 ml Buffer PB or add 600 µl pH indicator I to 150 ml Buffer PB). The yellow color of Buffer PB with pH indicator I indicates a pH of ≤ 7.5.

4. Add pH indicator I to entire buffer contents. Do not add pH indicator I to buffer aliquots.

5. If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without the addition of pH indicator I.

Procedure

Note: The PCR rxn was 100 µl. Therefore, need to add **500 µl of PB buffer**.

1. Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix. ***If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.***

2. Place a QIAquick spin column in a provided 2 ml collection tube.

3. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s. ***Discard flow-through and place the QIAquick column back into the same tube.***

4. To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s. ***Discard flow-through and place the QIAquick column back in the same tube.***

5. Centrifuge the column for an additional 1 min.

6. Place each QIAquick column in a clean 1.5 ml microcentrifuge tube.

7. To elute DNA, add **51 µl** Buffer EB (10mM Tris-Cl, pH 8.5) or water to the center of the QIAquick membrane and centrifuge the column for 1 min.

8. Check column to make sure there's no droplet stuck on the column. If there is still liquid, use a P10 pipet to suck up the droplet and add to the membrane. Spin an additional minute.

9. Discard column and save 1 µl for the Fluorescent Nanodrop reading (Picogreen).

Samples	Vol	Conc [ng/µl]	Total

V. Perform End Repair

This process converts the overhangs into blunt ends using T4 DNA polymerase and Klenow DNA polymerase. The 3' to 5' exonuclease activity of these enzymes removes 3' overhangs and the polymerase activity fills in the 5' overhangs.

A. End Repair

Illumina-Supplied	User-Supplied
10X End Repair Buffer	QIAquick PCR Purification Kit
25 mM dNTP Mix	(QIAGEN, part # 28104)
T4 DNA Polymerase	
Klenow DNA Polymerase	
T4 PNK	
Ultra Pure Water	

Procedure

1. Preheat one heat block to 20°C and the other heat block to 37°C. ***Note: We use the thermal cycler instead.***

2. Prepare the following reaction mix in a 200 μl thin-walled PCR tube (Total volume should be 100 μl):

Eluted DNA	-	50.0 μl
Water	-	27.4 μl
10X End Repair Buffer	-	10.0 μl
25 mM dNTP Mix	-	1.6 μl
T4 DNA Polymerase	-	5.0 μl
Klenow DNA Polymerase	-	1.0 μl
T4 PNK	-	5.0 μl

3. Incubate the sample in a PCR machine at 20°C for 30 minutes.

Note: Program name on the BioRad Mycycler PCR machine: **RNASeq End Repair**

Follow the instructions in the QIAquick PCR Purification Kit to purify the sample and elute in 33 μl of QIAGEN EB buffer.

B. Purify the cDNA using the Qiagen Purification Kit

This protocol is designed to purify single- or double-stranded DNA fragments from PCR and other enzymatic reactions. Fragments ranging from 100 bp to 10 kb are purified from primers, nucleotides, polymerases, and salts using QIAquick spin columns in a microcentrifuge.

Procedure

1. Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix. ***If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.***

Note: The PCR rxn was 100 µl. Therefore, need to add **500 µl of PB buffer**.

2. Place a QIAquick spin column in a provided 2 ml collection tube.

3. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s. ***Discard flow-through and place the QIAquick column back into the same tube.***

4. To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s. ***Discard flow-through and place the QIAquick column back in the same tube.***

5. Centrifuge the column for an additional 1 min.

6. Place each QIAquick column in a clean 1.5 ml microcentrifuge tube.

7. To elute DNA, add **33 µl** Buffer EB (10mM Tris-Cl, pH 8.5) or water to the center of the QIAquick membrane and centrifuge the column for 1 min.

8. Check column to make sure there's no droplet stuck on the column. If there is still liquid, use a P10 pipet to suck up the droplet and add to the membrane. Spin an additional minute.

9. Discard column and save 1 µl for the Fluorescent Nanodrop reading (Picogreen).

Samples	Vol	Conc [ng/µl]	Total

VI. Adenylate 3' Ends

This process adds an 'A' base to the 3' end of the blunt phosphorylated DNA fragments, using the polymerase activity of Klenow fragment (3' to 5' exo minus). This prepares the DNA fragments for ligation to the adapters, which have a single 'T' base overhang at their 3' end.

A. 3'End Adenylation

Illumina-Supplied
A-Tailing Buffer
1 mM dATP
Klenow exo (3' to 5' exo minus) (3 µl)

User-Supplied
MinElute PCR Purification Kit
(QIAGEN, part # 28004)

Procedure

1. Prepare the following reaction mix in a 200 µl RNase-free non-sticky tube (Total volume should be 50 µl):

Eluted DNA	-	32 µl
A-Tailing Buffer	-	5 µl
1 mM dATP	-	10 µl
Klenow exo (3' to 5' exo minus)	-	3 µl

2. Incubate the sample in a PCR machine at 37°C for 30 minutes.

Note: Program name on the BioRad Mycycler PCR machine: **RNASeq A Tail**

Follow the instructions in the MinElute PCR Purification Kit to purify the sample and elute in 24 µl of QIAGEN EB buffer.

B. Purify cDNA Using the Qiagen MinElute PCR Purification Kit

This protocol is designed to purify double-stranded DNA fragments from PCR reactions resulting in high end-concentrations of DNA (see page 12). Fragments ranging from 70 bp to 4 kb are purified from primers, nucleotides, polymerases, and salts using MinElute spin columns in a microcentrifuge.

Note: We've used a regular column (by accident) instead of a MinElute column and were able to recover sufficient cDNA.

Important points before starting

1. Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).

2. All centrifugation steps are carried out at 17,900 x g (13,000 rpm) in a conventional tabletop microcentrifuge at room temperature.

3. Add 1 : 250 volume pH indicator I to Buffer PB (i.e., add 120 µl pH indicator I to 30 ml Buffer PB or add 600 µl pH indicator I to 150 ml Buffer PB). The yellow color of Buffer PB with pH indicator I indicates a pH of ≤ 7.5.

4. Add pH indicator I to entire buffer contents. Do not add pH indicator I to buffer aliquots.

5. If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without the addition of pH indicator I.

Procedure

Note: The PCR rxn was 50 µl. Therefore, need to add **250 µl of PB buffer**.

1. Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix. ***If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.***

2. Place a MinElute spin column in a provided 2 ml collection tube.

3. To bind DNA, apply the sample to the MinElute column and centrifuge for 1 min. ***Discard flow-through and place the MinElute column back into the same tube.***

4. To wash, add 0.75 ml Buffer PE to the MinElute column and centrifuge for 1 min. ***Discard flow-through and place the QIAquick column back in the same tube.***

5. Centrifuge the column for an additional 1 min at max. speed.

6. Place each MinElute column in a clean 1.5 ml microcentrifuge tube.

7. To elute DNA, add **24 µl** Buffer EB (10mM Tris-Cl, pH 8.5) or water to the center of the MinElute membrane, let stand for 1 min., and centrifuge for 1 min.

8. Check column to make sure there's no droplet stuck on the column. If there is still liquid, use a P10 pipet to suck up the droplet and add to the membrane. Spin an additional minute.

9. Discard column and save 1 µl for the Fluorescent Nanodrop reading (Picogreen).

Samples	Vol	Conc [ng/µl]	Total

VII. Ligate the Adapters

This process ligates adapters to the ends of the DNA fragments, preparing them to be hybridized to a single read flow cell. Libraries constructed with this kit can be sequenced on a PE flow cell and give PE reads, however Illumina's post-sequencing analysis does not support PE mRNA reads.

A. Adapter Ligation

Illumina-Supplied	**User-Supplied**
2X Rapid T4 DNA Ligase Buffer	MinElute PCR Purification Kit (QIAGEN, part # 28004)
PE Adapter Oligo Mix	
T4 DNA Ligase	

Procedure

1. Prepare the following reaction mix in a 1.5 ml RNase-free non-sticky tube (Total volume should be 50 µl):

Eluted DNA	-	23 µl
2X Rapid T4 DNA Ligase Buffer	-	25 µl
PE Adapter Oligo Mix	-	1 µl
T4 DNA Ligase	-	1 µl

2. Incubate the sample in at room temperature for 15 minutes.

3. Follow the instructions in the MinElute PCR Purification Kit to purify the sample and elute in 11 µl of QIAGEN EB buffer.

You can spin the column twice as long after QIAGEN PE removal to ensure complete ethanol removal.

Prepare a 50 ml, 2% agarose gel with distilled water and TAE so that there will be time for the gel to set. Final concentration of TAE should be 1X at 50 ml.

B. Purify cDNA Using the Qiagen MinElute PCR Purification Kit

This protocol is designed to purify double-stranded DNA fragments from PCR reactions resulting in high end-concentrations of DNA (see page 12). Fragments ranging from 70 bp to 4 kb are purified from primers, nucleotides, polymerases, and salts using MinElute spin columns in a microcentrifuge.

Procedure

1. Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix. ***If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.***

2. Place a MinElute spin column in a provided 2 ml collection tube.

3. To bind DNA, apply the sample to the MinElute column and centrifuge for 1 min. ***Discard flow-through and place the MinElute column back into the same tube.***

4. To wash, add 0.75 ml Buffer PE to the MinElute column and centrifuge for 1 min. ***Discard flow-through and place the QIAquick column back in the same tube.***

5. Centrifuge the column for an additional 1 min at max. speed.

6. Place each MinElute column in a clean 1.5 ml microcentrifuge tube.

7. To elute DNA, add **11 µl** Buffer EB (10mM Tris-Cl, pH 8.5) or water to the center of the MinElute membrane, let stand for 1 min., and centrifuge for 1 min.

8. Check column to make sure there's no droplet stuck on the column. If there is still liquid, use a P10 pipet to suck up the droplet and add to the membrane. Spin an additional minute.

9. Discard column and save 1 µl for the Fluorescent Nanodrop reading (Picogreen).

Samples	Vol	Conc [ng/µl]	Total

VIII. Purify the cDNA Templates

This process purifies the products of the ligation reaction on a gel to select a size range of templates for downstream enrichment.

A. Run Samples on a 2% Gel

User-Supplied
Certified Low-Range Ultra Agarose
50X TAE Buffer
Distilled Water
100 bp DNA Ladder
6X DNA Loading Dye
GeneCatcher Disposable Gel Excision Kit
QIAquick Gel Extraction Kit (QIAGEN, part # 28704)

Procedure

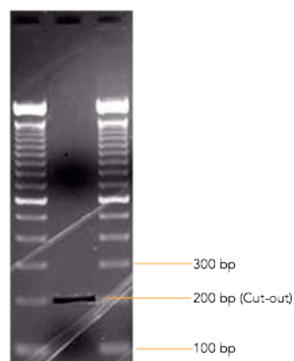
1. Prepare a 50 ml, 2% agarose gel with distilled water and TAE. Final concentration of TAE should be 1X at 50 ml. Leave one empty lane between samples and ladders to prevent cross- contamination. Do not run more than two samples on the same gel to avoid contamination.

2. Load the samples as follows:

Well 1	- 100 bp DNA Ladder	-	2 µl
Well 2	- 10 µl DNA with 2 µl 6X Xylene Cyanol Loading Dye	-	12 µl
Well 3	- 100 bp DNA Ladder	-	2 µl

3. Run the gel at 100 V for 80 minutes.

4. Excise a region of gel with a clean gel excision tip and remove the gel slice by centrifuging it into a microcentrifuge tube. The gel slice should contain the material in the 200 bp (±25 bp) range.



Follow instructions in the QIAquick Gel Extraction Kit to purify the sample and elute in 30 µl of QIAGEN EB buffer. (Be sure to add isopropanol per the manufacturer's instructions.)

B. Purify cDNA Using the Qiagen QIAquick Gel Extraction Kit

This protocol is designed to extract and purify DNA of 70 bp to 10 kb from standard or low-melt agarose gels in TAE or TBE buffer. Up to 400 mg agarose can be processed per spin column.

Important points before starting

1. Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
2. The yellow color of Buffer QG indicates a pH ≤ 7.5.
3. Isopropanol (100%) and a heating block or water bath at 50°C are required.
4. All centrifugation steps are carried out at 17,900 x g (13,000 rpm) in a conventional table-top microcentrifuge.

Procedure

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.

2. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl). For example, add 300 µl of Buffer QG to each 100 mg of gel.

3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2–3 min during the incubation.

4. Add 1 gel volume of isopropanol to the sample and mix.

5. Place a QIAquick spin column in a provided 2 ml collection tube.

6. To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min.

7. Discard flow-through and place QIAquick column back in the same collection tube.

8. Recommended: Add 0.5 ml of Buffer QG to QIAquick column and centrifuge for 1 min.

9. To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min.

11. Discard the flow-through and centrifuge the QIAquick column for an additional 1 min at 17,900 x g (13,000 rpm).

12. Place QIAquick column into a clean 1.5 ml microcentrifuge tube.

13. Elute DNA with **38 µl** of Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min.

14. Check column to make sure there's no droplet stuck on the column. If there is still liquid, use a P10 pipet to suck up the droplet and add to the membrane. Spin an additional minute.

15. Discard column and save 1 μl for the Fluorescent Nanodrop reading (Picogreen).

IX. Enrich the Purified cDNA Templates

This process uses PCR to amplify the cDNA in the library. The PCR is performed with two primers that anneal to the ends of the adapters.

A. Amplify the adapter-ligated cDNAs

Illumina-Supplied	**User-Supplied**
5X Phusion Buffer (Finnzymes Oy)	QIAquick PCR Purification Kit
Phusion DNA Polymerase (Finnzymes Oy)	(QIAGEN, part # 28104)
PCR Primer PE 1.0	
PCR Primer PE 2.0	
25 mM dNTP Mix	
Ultra Pure Water	

Procedure

1. Prepare the following PCR reaction mix in a 200 µl thin wall PCR tube (Make 10% extra reagent for multiple samples, Total volume should be 13 µl):

5X Phusion Buffer	-	10.0 µl
PCR Primer PE 1.0	-	1.0 µl
PCR Primer PE 2.0	-	1.0 µl
25 mM dNTP Mix	-	0.5 µl
Phusion DNA Polymerase	-	0.5 µl

2. Add 37 µl of purified ligation mix (from step 13 of the previous section) to the 200 µl PCR tube.

3. Amplify using the following PCR process:

    30 seconds at 98°C
    15 cycles of:
        10 seconds at 98°C
        30 seconds at 65°C
        30 seconds at 72°C
    5 minutes at 72°C
    Hold at 4°C

4. Follow the instructions in the QIAquick PCR Purification Kit to purify the sample and elute in 30 µl of QIAGEN EB buffer.

5. Validate the library by loading 1 µl of DNA on Bio-Rad Experion DNA chip or an Agilent BioAnalyzer. *Alternatively, load 1 µl of DNA on a 2% agarose gel and visualized the amplified band on the gel. Use 10,000X SYBR green gel stain to make the gel and add 5 µl to the electrophoresis buffer. Run the gel at 100V for 90 minutes. It is OK to use an undiluted 50 bp ladder.*

Check the size, purity, and concentration of the sample. The final product should be a distinct band at ~ 275 bp.

6. Aliquot the library to avoid repeated freeze/thaw cycles. Store the library in the -20°C freezer.

B. Purify cDNA Using the Qiagen PCR Purification Kit

This protocol is designed to purify single- or double-stranded DNA fragments from PCR and other enzymatic reactions. Fragments ranging from 100 bp to 10 kb are purified from primers, nucleotides, polymerases, and salts using QIAquick spin columns in a microcentrifuge.

Procedure

1. Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix. ***If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.***

2. Place a QIAquick spin column in a provided 2 ml collection tube.

3. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s. ***Discard flow-through and place the QIAquick column back into the same tube.***

4. To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s. ***Discard flow-through and place the QIAquick column back in the same tube.***

5. Centrifuge the column for an additional 1 min.

6. Place each QIAquick column in a clean 1.5 ml microcentrifuge tube.

7. To elute DNA, add **30 µl** Buffer EB (10mM Tris-Cl, pH 8.5) or water to the center of the QIAquick membrane and centrifuge the column for 1 min.

8. Check column to make sure there's no droplet stuck on the column. If there is still liquid, use a P10 pipet to suck up the droplet and add to the membrane. Spin an additional minute.

9. Use 1 µl for the Nanodrop reading.

X. Aliquot the Library

It is extremely important to aliquot the library to avoid the degradation caused by freeze/thaw cycles.

Appendix: Illumina Primers

This list of primers was obtained from Illumina Customer Support by Anhthu Bui through email communications. The primer sequences used for mRNA-Seq are listed below:

Paired End DNA oligonucleotide sequences PE Adapters1

```
5' P-GATCGGAAGAGCGGTTCAGCAGGAATGCCGAG 3′
5'  ACACTCTTTCCCTACACGACGCTCTTCCGATCT 3′
```

This double-stranded DNA adapter have a phosphorylated 5’ end (indicated by the “P”) and forms a Y shape with 13 base pairings. The overhang T in the 3’ end is used for ligation with the 3’ overhang A added to the cDNA.

```
5' P-GATCGGAAGAGCGGTTCAGCAGGAATGCCGAG 3′
      |||||||||||||
3′  TCTAGCCTTCTCGCAGCACATCCCTTTCTCACA 5′
```

PE PCR Primer 1.01

5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT 3′

The underlined sequence corresponds to the PE Read 1 sequencing primer and the non-phosphorylated strand of the adapter. The non-underlined sequence could represent sequences used to attach to the flow cell for cluster amplification.

PE PCR Primer 2.01

5'CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT 3′

The underlined sequence corresponds to the PE Read 2 sequencing primer. The non-underlined sequences could represent sequences used to attach to the flow cell for cluster amplification.

PE Read 1 Sequencing Primer

5'ACACTCTTTCCCTACACGACGCTCTTCCGATCT 3′

PE Read 2 Sequencing Primer

5'CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT 3′

Quality Analysis of RNA Samples Using the Bio-Rad Experion System – short version

This protocol is obtained from the original protocol written by Anhthu. It was shortened by Kelli on February 17, 2011.

Reference: Modification of a brief protocol from Bio-Rad Tech Support personnel, Quynh Trinh, sent to Anhthu on June 5, 2006.

Experion RNA **StdSens** Analysis kit is suitable for 1 µl samples with a concentration of 5-500 ng/ µl, up to 12 samples.
Experion RNA **HighSens** Analysis kit is suitable for 1 µl samples with a concentration of 100-5000 pg/ µl, up to 11 samples.

PROCEDURE

A. Cleaning the Electrodes BEFORE a Run

Minor modification by Anhthu Bui – November 22, 2006. This procedure should be performed daily. However, if RNase contamination is suspected, this procedure should be performed between each run.

1. Pour into an RNase-free 1.5-mL microfuge tube about **900 µl** of **Experion Electrode Cleaner solution** from a bottle on the shelf above the laptop driving the Experion.

2. Fill a cleaning chip with **800 µl Experion electrode cleaner** using a **P1000 pipetman**. Check to make sure there are no air bubbles trapped in the reservoir. **Gently tap** the side of the cleaning chip to dispel any bubbles. **Label** this **cleaning chip as "electrode cleaning"** and **Date**.

Note: If this cleaning chip is being used for the first time, treat it with the Experion electrode cleaner to remove any RNase contamination before use.

   a. Fill a new cleaning chip with **800 µl Experion electrode cleaner**. Let it sit for **5 minutes**.
   b. Wash the chip by adding **800 µl DEPC-treated water** and letting it sit for **1 minute.**
   c. Repeat the DEPC-treated water wash 4 more times.

3. Open the lid of the electrophoresis station and place the chip on the platform.

4. Close the lid and leave the chip in the instrument for **1 minute**. Caution: Do NOT leave electrodes in the solution for more than **2 minutes.** It is **safer** to **clean** the **electrodes** in **less than 2 minutes**.

5. Fill **another cleaning chip** with **800 µl DEPC-treated water** using a **P1000 pipetman**. Label this cleaning chip as "**DEPC water**" and **Date**.

6. Open the lid and remove the cleaning chip containing the electrode cleaner; replace it with the chip containing the DEPC-treated water.

7. Close the lid and leave the chip in the instrument for **1 minute** to **rinse the electrodes**. *Note: The **original protocol** states **5 minutes**. Anhthu thought it would be more efficient to remove detergents from the electrodes with more rinses with DEPC'd water.*

8. **Replace** the **DEPC-treated water** in the DEPC water chip and **repeat the rinse** step for 60 seconds for a total of 3 times.

9. **Open the lid** and **remove** the DEPC water chip.

10. Leave the lid open and wait about **30–60 seconds** for any water remaining on the electrodes to evaporate. When finished for the day, **discard** liquid from the cleaning chips.

B. Preparing the Gel Stain

1. Remove the RNA gel, RNA stain, loading buffer, and sensitivity enhancer from storage in refrigerator and allow them to equilibrate to room temperature for ~15 minutes in a drawer. Keep the RNA stain protected from light.

2. Briefly vortex the RNA stain (blue cap) and spin down in a microcentrifuge for 3–5 seconds. Make sure the DMSO in the stain is completely thawed.

3. Pipet 600 µl RNA gel solution (green cap) into a spin-filter tube.

4. Centrifuge the gel at 1500 x g for 10 minutes. Confirm that all of the gel has passed through the filter and then discard the filter. Note: Use the filtered gel within 4 weeks of preparation. After 4 weeks, the filtered gel should be refiltered and can be reused. *Note: Anhthu used a 4.5 week-old filtered gel solution and it was still good. Generally, after 4 weeks, the filtered gel should be re-filtered and can be reused.*

5. Pipet 65 µl filtered gel into a 1.5-mL brown RNase-free microcentrifuge tube. Then, pipet 1 µl RNA stain to that tube. Briefly vortex the solution. Keep the gel-stain solution protected from light. *This gel stain solution is enough for 3 chips.*

6. Cap the RNA stain tube tightly, since DMSO is highly hygroscopic, and store it in the dark in the kit box.

7. Centrifuge the gel-stain solution at 13,000 x g for 10 minutes. *Note: Anhthu found that 3 minutes of centrifugation at 13,000 rpm is sufficient.* Protect the gel-stain solution from light.

8. For best results, a new tube of gel stain should be prepared immediately before running each chip. *Note: Anhthu found that the gel-stain solution is good for 7-10 days.*

C. Preparing RNA Samples for Loading

1. Label **1.5-mL microfuge tubes** according to the RNA samples.

2. Pipet **1 µL** of **RNA sample** into 1.5-mL microfuge tubes.

3. Prepare RNA ladder.

   For StdSens:

   a. Pipet **1 µL** of **StdSens RNA ladder** into a 1.5-mL microfuge tube. *The StdSens RNA ladder is aliquoted into 2 µl aliquots stored at -70°C.*

   b. Denature the **StdSens ladder** and **sample RNA** tubes at **70°C** for **2 minutes**. *It is okay to incubate for 3-5 minutes.*

   c. Quench **on ice** for **5 minutes or longer**.

   d. Spin tubes in the **microfuge** for **15-30 seconds**. Put tubes back **on ice**.

For HighSens:

a. Pipet **1 µL** of **diluted HighSens RNA ladder** into a 1.5-mL microfuge tube. *Note: 5 µl ot the HighSens RNA ladder has been denatured for 2 minutes at 70°C, cooled for 5 minutes on ice, spun down, diluted with 795 µl of DEPC-treated water (0.2 µm filtered) and aliquoted into 5 µl aliquots stored at -70°C. Avoid exposing the diluted RNA ladder to freeze-thaw cycles.* ***Do not incubate the aliquoted HighSens RNA ladder at 70°C. Keep the ladder on ice.***

b. Denature the **sample RNA** tubes at **70°C** for **2 minutes**. *It is okay to incubate for 3-5 minutes*.

c. Quench **on ice** for **5 minutes or longer**.

d. Spin tubes in the **microfuge** for **15-30 seconds**. Put tubes back **on ice**.

D. Preparing a Chip

1. Pipet **9** µ**L** of **GS (gel + staining dye) solution** to the **bottom** of the **highlighted GS** well (*third well from the top*).
2. Prime the chip on the **Priming Station** using **B1** setting. *It takes 30 seconds*.
3. Pipet another **9** µ**L** of **GS solution** to the **bottom** of the other **GS** well (*second well from the top*).
4. Pipet **9** µ**L** of **purified Gel solution** into the **G** well (*top well*).
5. Pipet **6** µ**L** of **ES (Enhanced Solution,** tube with **CLEAR cap)** into the **ES** well. *Note: This step is ONLY required for the HighSens chip.*

E. Loading RNA Mixture into Wells

1. Pipet **5** µ**L** of **Loading Buffer** (tube with **YELLOW cap**) into each RNA solution, including the RNA ladder solution. Mix by flicking. Set the RNA mixture on ice until loading. *The total volume of the RNA mixture is ~6 µL*.
2. Pipet **6** µ**L** of **RNA Ladder** to **well "L."**
3. Pipet **6** µ**L** of **RNA mixtures** to **wells** 1-11 for HighSens chip or 1-12 for StdSens chip. *All wells on the chip must be loaded with a sample or 6 µL of loading buffer.*
4. Inspect **the wells** for **any bubbles**. *If there are bubbles in the wells, give one or two gentle taps of the chip on the bench*.
5. **Run** the chip **immediately** within 5 minutes of loading all the samples.

F. Performing the Analysis Run

1. Put the chip on the Experion **analyzer**.
2. Start the analysis program by pressing the "**PLAY**" symbol button. *It takes about 25 and 27 minutes for analyzing the HighSens chip (11 samples) and StdSens chip (12 samples), respectively*.

3. A window will popup asking for the total number of samples loaded. Indicate the number of samples and the software will avoid the wells containing loading buffer.

4. At the end of the analysis, the **analyzer beeps**.

5. Replace the sample chip with the **chip containing 800 µL of DEPC'd water** to rinse the probes attached to the lid.

6. Leave it in there for **1-2 minutes**.

7. Remove the chip and let the **probes dry**.

8. Close the lid of the analyzer.

9. Turn off the analyzer.

10. Close the Experion program after analysis is done.

Quality Analysis of DNA Using the Bio-Rad Experion System

This protocol was written by Kelli on November 8, 2010. Modified December 20, 2010

Reference: Experion DNA 1K Analysis Kit Instruction Manual

The Experion 1K analysis kit can perform DNA electrophoresis in the range between 25 and 1,000 bp. This kit uses lower (15 bp) and upper (1,500 bp) internal markers for alignment. *: DNA samples must be within the linear range of the assay (0.1-50 ng/μl). Dilute samples in TE buffer as needed.*

PROCEDURE

It is not necessary to clean the electrodes before running a DNA chip.

7. Preparing the Gel Stain

1. Remove the kit containing the DNA gel, DNA stain and loading buffer from storage and allow them to equilibrate to room temperature for ~15 minutes in a drawer. Keep the DNA stain protected from light. Put the DNA ladder (clear cap) on ice.

2. Invert the tubes several times, vortex the contents and briefly centrifuge.

3. Pipet **12.5 μl DNA stain** to a tube of **250 μl DNA 1K gel** (green cap). Vortex 10 sec. Centrifuge briefly.

4. Transfer all of the gel-stain solution to a spin filter tube. Label and date the tube.

5. Centrifuge at 2,400 x g for 15 min. Confirm that all of the gel has passed through the filter and then discard the filter. Transfer gel-stain to an amber tube. *Note: Use the filtered gel within 4 weeks of preparation. After 4 weeks, discard the solution. (I used a 5 week old filtered gel and it was ok.) The filtered gel-stain should NOT be refiltered and used. Each tube of filtered gel-stain should contain sufficient solution for at least 5 chips.*

C. Preparing DNA Samples for Loading

1. The DNA 1K ladder (clear cap) should be kept on ice. Briefly centrifuge, and then vortex to mix.

   Put back on ice.

2. Label a **1.5-mL microfuge tube** "L" and pipet **1 μl DNA Ladder** into the tube.

3. Label **1.5-mL microfuge tubes** according to the DNA samples. *Note: DNA samples must be within the linear range of the assay (0.1-50 ng/μl). Dilute samples in TE buffer as needed.*

4. Pipet **1 μl DNA sample** into each sample tube.

D. Preparing a DNA Chip

1. Pipet **9 μL** of **GS (gel + staining dye) solution** to the **bottom** of the **highlighted GS** well (*third well from the top*).

2. Prime the chip on the **Priming Station** using **C3** setting. Turn the chip over and inspect the microchannels for bubbles or evidence of incomplete priming. The microchannels will appear

opaque and will be difficult to see if the chip is primed properly. If you see a problem, prime a new chip.

3. Pipet **9 µL GS solution** to the **bottom** of the other 3 **GS** wells.

E. Loading DNA Samples and Ladder

1. Pipet **5 µL** of **Loading Buffer** (**yellow cap**) into each tube of 1 µl DNA sample or ladder. Mix by flicking. *The total volume of the DNA mixture is **~6** µ**L***.
2. Pipet **6 µL** of **DNA sample** to wells 1-11. *All wells on the chip must be loaded with a sample or buffer. If there are fewer than 11 samples to load on the chip, load the remaining wells with loading buffer.*
3. Pipet **6 µL diluted DNA Ladder** to **well "L"**.
4. Inspect **the wells** for **any bubbles**. *If there are bubbles in the wells, give one or two gentle taps of the chip on the bench*.
5. Run the chip immediately within 5 minutes of loading all the samples.

F. Performing the Analysis Run

1. Put the chip on the Experion **analyzer**.
2. Select **New Run**. Select **DNA 1K** protocol from the dropdown menu. Enter project name.
3. Start the analysis program by pressing the "**PLAY**" symbol button. *The analysis takes about 30 min.*
4. A window will popup asking for the total number of samples loaded. Indicate the number of samples and the software will avoid the wells containing loading buffer.
5. At the end of the analysis, the **analyzer beeps**.
6. Replace the sample chip with the **chip containing 800 µL of DEPC'd water** to rinse the probes attached to the lid.
7. Leave it in there for **1 minute**.
8. Remove the chip and let the **probes dry** for **1 minute**.
9. Close the lid of the analyzer.
10. Turn off the analyzer.
11. Close the Experion program after analysis is done.

RIBOGREEN ASSAY USING NANODROP ND-3300 FLUOROSPECTROMETER (Updated January 19, 2011)

Reference:

RiboGreen Assay for RNA Protocol (Thermo Scientific - attached, you can also find this on the webbook or in Lab Files).

Jones *et al*. RNA Quantitation by Fluorescence-Based Solution Assay: RiboGreen Reagent Characterization. Anal Biochem. 1998 Dec 15;265(2):368-74.

Attention:

Read the attached Thermo Scientific protocol because it contains detailed information and cautions.
This protocol is written based on the Thermo Scientific protocol with modifications from Kelli and Anhthu.
Practice RNase-free.

Reagents and Materials needed:

1. **Quant-iT** (Invitrogen, R11490) kit contains the following:
    a. **20x TE buffer** stored at room temperature on the shelf for RNA reagents
    b. **RiboGreen reagent** stored in aliquots of **5 µL/tube** in an RNase-free **-20°C freezer**. *Caution: RiboGreen is light sensitive!*
    c. **RNA standard** (**100 ng/µL**; ***E. coli*** **ribosomal RNA**) stored at **-80°C**.
2. **Autoclaved DEPC-treated water**
3. RNase-free **AMBER brown** or foil-covered 1.5 mL microcentrifuge tubes
4. RNase-free pipet tips
5. Pipetman sets (P-2 or P-10, P-100, P-200 and P-1000)
6. 14-mL RNase-free centrifuge tube
7. Microcentrifuge tube rack
8. A rack for 14-mL centrifuge tube or a 150-mL glass beaker to hold the tube
9. A Revco cardboard box for holding microfuge tubes (only if clear tubes are used)
10. A box of Kimwipes
11. Vortex mixer
12. Microcentrifuge
13. RNase-Zap (Ambion, Cat. #9786) or Freshly prepared DEPC treated water
14. Aluminum foil for covering the microfuge rack with tubes

PROCEDURE

A. MEASUREMENT OF EXPERIMENTAL SAMPLES

<u>Note:</u>

- *Standard curves must be generated and saved before the measurement of experimental samples proceeds. If they are not generated, please follow section B. However, if standard curves are already generated, then proceed with the protocol.*
- *Nanodrop recommends diluting the UNKNOWN RNA with 1x TE buffer at an estimated RNA concentration that will fall in the middle of the standard curve. However, you do not know the concentration of your RNA samples.* ***What can you do?***

1. Prepare 1x **TE buffer** for **diluting** the **RiboGreen stock** solution (**aliquot of 5 µL**) and **RNA samples** as following:

	1.5 mL	**2 mL**	**3mL**
20X TE buffer stock	75 µL	100 µL	150 µL
Autoclaved DEPC-treated water	1,425 µL	1,900 µL	2,850 µL

   Vortex briefly to mix the contents.
   Spin briefly.

2. Prepare the appropriate **diluted RiboGreen solution** according to the standard curve that you will use to measure the concentration of your samples.

Range of the final concentration of the sample when measured	**Standard curve**	**RiboGreen dilution**
0.625 ng/µL to 10ng/µL	HIGHEST	1:20
25 pg/µL to 1,000 pg/µL	HIGH	1:200
5 pg/µL to 50 pg/µL	LOW	1:2000

3. Remove **ONE** aliquot (**5 µL)** of **RiboGreen** solution from the Revco cardboard box labeled "**RiboGreen** Stock" in the **–20°C RNA freezer**. Thaw the tube briefly at room temperature. Flick the tube gently to mix the contents.

a. Spin briefly.

b. Add **1x TE buffer** to achieve the proper dilution.

Standard curve	**RiboGreen dilution**	**Volume of 1x TE buffer added to 5 µL RiboGreen**
HIGHEST	1:20	95 µL
HIGH	1:200	995 µL
LOW	1:2000	*

* To make a 1:2000 dilution for LOW, first prepare a 1:200 dilution as you for the HIGH standard curve. Then make a 1/10 dilution of that by mixing 100 µL of 1:200 dilution and 900 µL of 1x TE.

c. Vortex briefly to mix.

d. Spin briefly.

e. Place tube in microfuge tube rack. <u>Note:</u> *If clear tubes with aluminum foil covers are used, use a piece of aluminum foil to cover the microfuge tube rack or put tubes in a Revco box to avoid exposing **RiboGreen** dye to light. Alternatively, use AMBER tubes.*

4. Label 1.5-mL RNase-free **AMBER** microfuge tubes according to the RNA samples and one tube of "Reference." Set tubes on a microfugetube rack.
5. Pipet **3 µL** of **1x TE buffer** into each of the appropriate amber sample tubes, and **4 µL** of **1x TE buffer** into the **"Reference"** tube.
6. Pipet **1 µL** of **RNA solution** into the appropriate amber sample tubes. Mix by pipetting up and down 5 times. <u>Note:</u> *The total volume is **4 µL**.*
7. Pipet **4 µL** of the **appropriate dilution of RiboGreen** to the "Reference" tube. Mix by pipetting up and down for 5 times or flicking the tube to mix the contents.
8. Repeat step 7 for each tube of RNA sample.
9. After you have finished adding diluted **RiboGreen** solution to all RNA samples. Spin tubes briefly in a microfuge. <u>Note:</u> *If you are not using amber tubes, **immediately** place the tube in the Revco cardboard box or on a microfuge tube rack that is covered with a piece of aluminum foil.*

10. **Incubate** the mixtures for **5 minutes** at room temperature. <u>Note:</u> *It is okay to incubate for more than 5 minutes*.
11. Open the arm of the Nanodrop Fluorospectrometer or "Fluorodrop." Clean the pedestals with a piece of Kimwipes wetted with either **freshly prepared** DEPC-treated water (NOT autoclaved) or RNase-Zap solution. **Blot** (**NOT** swipe) off excess liquid with another piece of Kimwipes.
12. Pipet **1.5 µL** of **autoclaved DEPC-treated water** on the LOWER pedestal. Close the arm so the upper bushing interfaces with the water droplet.
13. Open the arm and blot off the water from both surfaces with a piece of Kimwipes.
14. Open the **ND-3300 software** by clicking on the **ND-3300 icon** on the desktop of the computer --> Click on the **Nucleic Acids** button --> Select **RiboGreen-RNA** from the pull down menu.
15. Pipet **1.5 µL** of **Reference solution** to the LOWER pedestal. Close the arm. Click the **BLANK** button. Blot off the Reference solution from the pedestals. <u>Note:</u> *Nanodrop and Anhthu recommend blanking with 1x TE and then measuring the fluorescence of the Reference as a negative control. If this approach is taken, the RFU reading of the reference should be in the range of 15-45.*
16. Upload ONE of the **three** saved **STANDARD** curves (HIGHEST, HIGH or LOW) by clicking on **STANDARD CURVES** pull down menu --> Selecting **LOAD** --> Selecting "**High Std Curve.nfs**" file from the Standard curves folder --> Click **OK** button.
17. Click on the **MEASUREMENT TYPE** box --> Select REFERENCE → Select the desired units (ng/µL for HIGHEST, pg/µL for HIGH or LOW).
18. Pipet **1.5 µL** of **Reference solution** to the LOWER pedestal. Close the arm. Click the **MEASURE** button. *The RFU reading should be 0 if the Reference solution was used as the Blank*.
19. Blot off the solution from the pedestal surfaces with Kimwipes.
20. Repeat reading Reference solution for the total of 3-5 times, each time with new 1.5 µL solution, NOT reading one droplet for 3-5 times.
21. Click on the **MEASUREMENT TYPE** box --> Select SAMPLES.
22. Enter sample **ID Information**, e.g. Soybean Epidermis.

23. Enter the dilution factor. If you prepared your sample with 1 μL of RNA, 3 μL of 1x TE and 4 μL of diluted **RiboGreen**, then the dilution factor is 8.
24. Pipet **1.5 μL** of **RNA sample solution** to the LOWER pedestal. Close the arm. Click the **MEASURE** button. <u>Note:</u> *Pay attention to a read-out concentration of the RNA sample. This is the concentration of an 8-fold diluted RNA solution.*
25. Blot off the solution from the pedestal surfaces.
26. Repeat reading RNA solution for the total of **3-5 times**, each time with new 1.5 μL solution, NOT reading one droplet for 3-5 times.
27. After all concentration readings for all experimental samples are done, click on the **SHOW REPORT button** --> Select File pull down menu --> Select SAVE WINDOW --> Type in Name of the file in the FILE NAME field --> Click OK button. <u>Note:</u> *You also want to print out the report by Selecting PRINT WINDOW from the File pull down menu. The print out will display the **actual concentration** of your RNA sample, not the 8-fold diluted concentration.*

<u>*Attention:*</u>

*What happens if the loaded standard curve is not appropriate for a certain RNA samples? There is a **warning message** saying that **the concentration is out of range**.*

<u>*Anhthu's suggestion:*</u>

***Do NOT** blot off the RNA sample from the pedestals. Upload the **HIGHEST** (if you suspect that concentration is higher) or **LOW** (if you suspect that concentration is lower) standard curve from the PULL down menu of STANDARD CURVES.* <u>*Note:*</u> *Anhthu always used 1:200 dilution of RiboGreen (even for the HIGHEST and LOW standard curves). This is not recommend by NanoDrop.*

<u>*Kelli's suggestion:*</u>

If the RNA sample is too concentrated, either use the NanoDrop 1000 or dilute the sample with 1x TE and the appropriate dilution of RiboGreen. Change the dilution factor. Upload the appropriate standard curve.

IF *you forgot to report your dilution factor before measuring your samples, then you need to calculate the actual concentration of RNA samples by multiplying the read concentration with the dilution factor (fold of dilution with 1x TE buffer and diluted RiboGreen solution). How? Open the Hard drive C → the ND3300 folder → the Default folder → RiboGreen folder → Select the file of your RNA concentration → Right-click and select OPEN WITH Excel → Copy relevant columns up to the column with Units and all rows with samples → Paste the copied information to a NEW Excel file → Calculate RNA concentration and yield as exemplified below*

Concentration and Yield of RNA Isolated from Soybean Leaves in Paraffin Mixtures

Method: **RiboGreen - RNA**
Software: 2.5.0

Sample ID	Standard Curve	Date	Conc. (pg/uL)	Average Conc. (pg/uL)	Dilution Factor	Calculated Conc. (ng/uL)	Volume of RNA (uL)	Amount of RNA (ng)
Blank	High	11/3/06	0					
Reference	High		0					
			0					
Soy Leaf1 Paraffin1	High		141.28	138.1	4	0.552	20	11.0
			134.87					
Soy Leaf2 Paraffin1	High		584.28	585.2	4	2.34	20	46.8
			586.1					

B. PREPARATION OF HIGHEST, HIGH AND LOW RNA STANDARD CURVES

Note:

- ***Once standard curves are created and saved, they do not need to be generated again until a new lot of RiboGreen is used**.*
- *The Nanodrop protocol suggests preparing only **two ranges (HIGH** of 25 pg/µL to 1000 pg/µL and LOW of 5 pg/µL to 50 pg/µL) of standard curves. We, the Goldberg lab, wanted to get the HIGHEST (0.625 ng/µL to 10ng/µL) standard curve to link the concentration in the HIGH range of RNA at 1,000 pg/µL determined by Fluorospectrometer and that (greater than 1000 pg/µL) determined by the Nanodrop ND-1000 UV spectrophotometer.*
- *All works are done under normal light conditions in the room. That is, NO light is turned off.*
- *Invitrogen's **RiboGreen** kit comes with an **RNA standard (100 ng/µL**; **E. coli ribosomal RNA**) stored at **-80°C**. However, any clean RNA free of genomic DNA contamination can be used as the RNA stock. I (Kelli) have used RiboAmp Control RNA from Arcturus as an RNA stock.*

1. Prepare the amount of **1x TE buffer** that you need from **20x TE buffer** in a 14-mL RNase-free tube.

	1.5 mL	**2 mL**	**3mL**
20X TE buffer stock	75 µL	100 µL	150 µL
Autoclaved DEPC-treated water	1,425 µL	1,900 µL	2,850 µL

   Vortex to mix the contents. Spin briefly.

2. Prepare a 100 ng/µL solution of stock RNA. Confirm the concentration of RNA on the NanoDrop-1000. Check the A_{260}/A_{280} and A_{260}/A_{230} ratios.
3. Label FIVE 1.5-mL **AMBER** microfuge tubes for EACH standard curve as 1-5 for HIGHEST, H1-H5 for HIGH, and L1-L5 for LOW.
4. Prepare serially diluted solutions at 2x (twice the final) concentrations for standard curves.

   a. HIGHEST standard (0.625 ng/µL - 10 ng/µL)

Tube #	Stock RNA Conc. (ng/µL)	Volume of RNA Stock (µL)	Volume of 1x TE (µL)	2x RNA Std Conc. (ng/µL)	Final Conc. In Assay (ng/µL)
1	100	20	80	20	10
2	20	50	50	10	5
3	10	50	50	5	2.5
4	5	50	50	2.5	1.25
5	2.5	50	50	1.25	0.625

Note: For each diluted solution, vortex briefly and spin briefly.

- Discard 50 µL of solution in tube #**5**.
- Place tubes on the rack for microfuge tubes.

b. HIGH standard (25 pg/µL - 1,000 pg/µL)

- Prepare a **2ng/µL (or 2000 pg/µL) RNA solution** from the concentrated RNA stock

$$\frac{(100\ \text{ng}/\mu\text{L})(3\ \mu\text{L})}{2\ \text{ng}/\mu\text{L}} = 150\ \mu\text{L total volume}$$

Vol. of 1x TE = 150 µL - 3 µL = 147 µL

Vortex briefly. Spin briefly.

Tube #	Stock RNA Conc. (pg/µL)	Volume of RNA Stock (µL)	Volume of 1x TE (µL)	2x RNA Std Conc. (pg/µL)	Final Conc. In Assay (pg/µL)
H1	2,000	50	0	2,000	1,000
H2	2,000	50	50	1,000	500
H3	1,000	50	50	500	250
H4	500	50	50	250	125
H5	250	10	40	50	25

Note: For each diluted solution, vortex briefly and spin briefly.

- Save the remaining volume of the 2ng/μL RNA solution for the preparation of LOW standard solutions.
- Discard 40 μL of solution in tube #**H4**.
- Place tubes on the rack for microfuge tubes.

c. LOW standard (5 pg/μL - 50 pg/μL)

- Prepare a **100 pg/μL RNA solution** from the 2ng/μL RNA dilution in step 4b.

(2 ng/μL)(10 μL) / (0.1 ng/μL) = 200 μL total volume

Vol. of 1x TE = 200 μL - 10 μL = 190 μL
Vortex briefly. Spin briefly.

Tube #	Stock RNA Conc. (pg/μL)	Volume of RNA Stock (μL)	Volume of 1x TE (μL)	2x RNA Std Conc. (pg/μL)	Final Conc. In Assay (pg/μL)
L1	100	50	0	100	50
L2	100	80	20	80	40
L3	80	50	50	40	20
L4	40	50	50	20	10
L5	20	50	50	10	5

Note: For each diluted solution, vortex briefly and spin briefly.

- Discard **50 μL** of solution in tube #**L5**.
- Place tubes on the rack for microfuge tubes.

5. Prepare **THREE diluted RiboGreen solutions**. 1:20 for HIGHEST, 1:200 for HIGH and 1:2000 for LOW.
   a. Remove **FIVE** tubes of the aliquot **(5 μL)** of **RiboGreen** solution from the -20°C RNA freezer.

b. Spin briefly.

c. Add **1x TE buffer** to achieve the proper dilution.

- For HIGHEST, add **95 µL** of **1x TE buffer** to each of **FOUR 5 µL RiboGreen aliquots.**
- For HIGH, add **995 µL** of **1x TE buffer** to ONE **5 µL RiboGreen aliquot.**
- For LOW, mix **100 µL** of **1:200 dilution** (from HIGH) **and 900 µL** of **1x TE buffer**.

d. Vortex briefly to mix.

e. Spin briefly.

6. Prepare the HIGHEST standard curve.

   a. Add **50 µL** of the **1:20 RiboGreen dilution** to each tube containing **50 µL** of the **HIGHEST** standard solutions. Vortex briefly. Spin briefly. *Note: Use **50 µL** of **1:200 RiboGreen dilution** for **HIGH** standard curve and **50 µL** of **1:2000 RiboGreen dilution** for **LOW** standard curve.*

   b. **Incubate** the mixtures for **5 minutes**. *Note: It is okay to incubate for more than 5 minutes*.

7. Prepare a **REFERENCE** solution as following:

   1x TE buffer 50 µL

   1:20 diluted **RiboGreen** solution 50 µL

   Vortex briefly. Spin briefly. **Incubate** for **5 minutes**. . *Note: It is okay to incubate for more than 5 minutes*.

   *Note: Use **50 µL** of **1:200 RiboGreen dilution** for **HIGH** standard curve and **50 µL** of **1:2000 RiboGreen dilution** for **LOW** standard curve.*

8. Open the **ND-3300 software** on the desktop of the computer --> Click the **Nucleic Acids** button --> Select **RiboGreen - RNA**.

9. Clean the sampling pedestals with a piece of Kimwipes wetted with either the RNAse-ZAP solution or freshly prepared DEPC-treated water.

10. Pipet **2 µL** of **autoclaved DEPC-treated water** on the LOWER pedestal. Close the arm so the upper bushing interfaces with the water droplet.

11. Open the arm and use Kimwipes to **BLOT away** (NOT wipe off) the water from both pedestal surfaces. Note: *Make sure that the optical surfaces are free of lint before proceeding.*
12. Pipet **2 μL** of **REFERENCE** solution to the LOWER pedestal. Close the arm. Click the **BLANK** button. Blot off the Reference solution from the pedestals. *Note: Nanodrop and Anhthu recommend blanking with 1x TE and then measuring the fluorescence of the Reference as a negative control. If this approach is taken, the RFU reading of the reference should be in the range of 15-45.*
13. Click the **MEASURMENT TYPE** drop box --> Select **REFERENCE** --> Select desired **units (pg/μL**).
14. Pipet another **2 μL** of the **REFERENCE solution** (0 pg/μL) onto the LOWER pedestal. Close the arm. Click the **MEASURE** button. *The RFU reading should be 0 if the Reference solution was used as the Blank.*
15. Blot off the solution from the pedestal surfaces with Kimwipes.
16. Repeat reading **REFERENCE** solution for the total of **3-5 times**, each time with a new 2 μL of solution, NOT reading one droplet for 3-5 times.
17. Click on the **MEASUREMENT TYPE** drop box --> Select **STANDARD 1**.
18. Enter the **FINAL ASSAY** concentration of **STANDARD1**.
19. Pipet **2 μL** of the **STANDARD 1 solution** onto the LOWER pedestal. Close the arm, and click the **MEASURE** button. Blot off the sampling pedestals.
20. Measure **3-5 replicates** for each diluted HIGHEST standard solutions. *Again, use fresh droplet of solution for each measurement*.
21. Once the standard curve is completed, save the HIGHEST STANDARD curve by selecting **STANDARD CURVES** pull down menu --> Select "**SAVE AS...**" --> Create a Folder for STANDARD CURVES --> Select that folder --> Type in "**HIGHEST Std Curve_date**" --> Click **OK** button. *Note: The saved standard curve will be loaded during the measurement of experimental samples*.
22. Repeat steps 6-21 for HIGH and LOW standard curves.
23. Print out the new standard curves and post on the wall.

At the end of this work, you have THREE standard curves for determination of concentrations of Experimental RNA samples. You don't need to generate NEW standard curves until a new lot of RiboGreen is used.

PROTOCOL NanoDrop 3300

RiboGreen® Assay for RNA Quantitation

The RiboGreen® dye is a fluorescent nucleic acid stain for quantitating intact RNA. Used in conjunction with the micro-volume capability of the Thermo Scientific NanoDrop™ 3300 Fluorospectrometer, the RiboGreen® assay provides a highly sensitive means of RNA quantitation with minimal consumption of sample. The main disadvantage of general UV spectroscopy for RNA quantitation is the contribution of signal from degraded RNA and other contaminants, such as proteins and extraction buffers. RiboGreen® reagent circumvents such contributions from interfering substances by exhibiting an emission maximum at 530nm when bound specifically to intact RNA (unbound RiboGreen® reagent exhibits minimal fluorescence in solution). The ability of the NanoDrop 3300 to measure as little as 1 ul of sample, allows significantly scaled-down reaction volumes, thereby using only a fraction of sample commonly needed for conventional cuvette-based fluorometers. The NanoDrop 3300 has demonstrated a detection range for RNA of 25 ng/ml - 1000 ng/ml when using a high concentration of RiboGreen® dye (1:200 dilution), and 5 ng/ml - 50 ng/ml when using a 1:2000 dilution of RiboGreen® dye. (The following protocol is an adaptation of the Molecular Probes™ RNA Reagent Kit product information sheet.)

RiboGreen® Assay Supplies

Equipment:

- NanoDrop 3300 Fluorospectrometer
- 2uL pipettor (low retention nuclease free pipette tips)

Materials:

- Low lint laboratory wipes
- Nuclease free amber or foil covered 1.5 ml polypropylene tubes
- RNase inhibitor wipe or solution
- Polypropylene bottles, vials, and tubes

Reagents:

- RiboGreen® RNA reagent kit (Molecular Probes catalog # R11490) Includes 20X TE.
- DEPC treated water

RiboGreen® Assay Suggestions

- Aliquot dye concentrate into amber screw top tubes. Store at -20°C.
- Wipe down bench space, pipettes, and racks with RNase inhibitor before starting assay.
- Change gloves often to minimize nuclease contamination.

RiboGreen® Assay Protocol

1. Equilibrate the 20X TE buffer, RiboGreen® reagent (200X concentrate and DEPC treated dH_2O to room temperature. Protect from light.
2. Prepare 1X TE with DEPC treated water. The volume needed depends on the total number of samples to be measured and the volume of RiboGreen working solution.
3. Dilute the RiboGreen® Dye stock in two stages depending on RNA standard concentration range. (*Prepare fresh as the diluted dye is stable for only a few hours. Protect all stocks from light.*

High RNA Quantitation range (25 ng/ml–1000 ng/ml)
- Dilute the RiboGreen® stock (200X concentrate) by transferring 995 ul of 1X TE and 5 ul of the dye stock to a 1.5ml amber snap cap tube. Mix thoroughly.

Low RNA quantitation range (5 ng/ml– 50 ng/ml)
– Dilute the 1X dye in the previous step ten (10) fold further by transferring 900ul of 1X TE and 100ul of the 1X dye dilution to a 1.5ml amber snap cap tube,. Mix thoroughly.

4. Thaw the standard and unknown RNA samples. Once thawed, thoroughly mix each individual solution gently .
5. Prepare serially diluted RNA standards to 2X final concentrations in nuclease free vials or tubes (please refer to the example standard curve dilution series on page 2).
6. Aliquot one volume of diluted unknown RNA samples into appropriately labeled nuclease free amber tubes.

   Note: It is recommended that RNAs be diluted into 1X TE at an estimated 2x concentration which will fall in the middle of the standard curve.
7. Transfer an equal volume of the appropriate diluted RiboGreen® dye solution to each amber tube containing either the 2X standard RNA solution or unknown sample.
8. Prepare the Reference solution (negative control) by adding equal volumes of 1X TE and RiboGreen® working solution.
9. Mix each standard dilution and unknown sample thoroughly and allow to equilibrate at room temperature for 5 minutes. Proceed to Standard Curve Protocol (page 2).

Standard Curve Protocol

1. Clean both sampling pedestals with 2 uL of nuclease free deionized water.
2. Open upper arm and firmly blot the two pedestals with a dry lab wipe. Make sure there are no traces of lint on the pedestals before continuing.
3. Open the operating software. Click on the Nucleic Acid Quantitation button and select the RiboGreen method.
4. Add 2 uL of assay buffer (no dye, no sample) to the lower pedestal. Lower the arm and click F3 or the Blank button. When the measurement is complete, lift the arm and use a dry laboratory wipe to blot the buffer from both the bottom and upper measurement surfaces. Use a fresh aliquot of buffer to verify a proper baseline.
5. Under Measurement type, click on the Standards tab. Highlight the Reference standard.
6. Mix the reference solution (assay buffer and dye, no sample) briefly and transfer 2 uL of the solution onto the lower pedestal. Lower the arm and click F1 or the Measure button. A pop up window will ask for confirmation of the units. (Recommended ng/mL or pg/uL)
7. Measure up to 5 replicates of the reference solution using a fresh 2 uL aliquot for each measurement.
8. Select Standard 1 to enter a value. Enter values for up to 7 standards.
9. Mix the standard solution briefly and transfer 2 uL onto the lower pedestal. Lower the arm and click F1 or the Measure button. Measure up to 5 replicates of each standard using a fresh 2 uL aliquot for each measurement.
10. Once the standard curve is completed, select the Standard Curve Type (Interpolation, Linear, 2° polynomial, 3° polynomial) that best fits the standards data set.
11. Click on the Sample tab under Measurement Type, and enter the unknown samples' respective ID information. If a dilution of the unknown sample was made, enter the dilution factor in the box below the sample ID window.
12. Add 2 ul of the sample and use the F1 key or click the Measure button to initiate the measurement cycle. Use a fresh aliquot of sample for each measurement.

High RNA concentration range

Example of standard curve dilution series

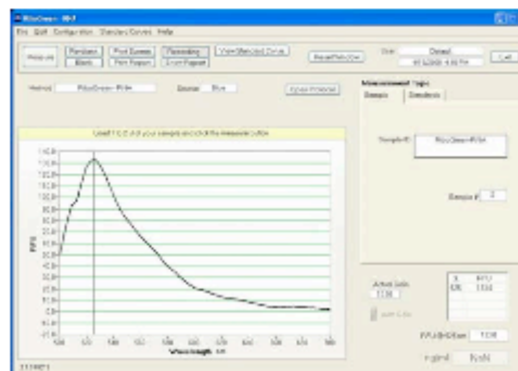
Stock RNA (ng/ml)	2X RNA volume (ul)	1X TE buffer volume (ul)	2X RNA standard (ng/ml)	Final RNA (ng/ml)
2000	10*	0	2000	1000
2000	10**	10	1000	500
1000	10	10	500	250
500	10	10	250	125
250	5	20	50	25

Low RNA concentration range.

Example of standard curve dilution series

Stock RNA (ng/ml)	2X RNA volume (ul)	1X TE buffer volume (ul)	2X RNA standard (ng/ml)	Final RNA (ng/ml)
100	10*	0	100	50
100	20**	5	80	40
80	10	10	40	20
40	10	10	20	10
20	10	10	10	5

Example spectrum of RiboGreen RNA sample



Rev 4/08

PICOGREEN ASSAY USING NANODROP ND-3300 FLUOROSPECTROMETER
(Updated January 19, 2011)

References:

PicoGreen Assay for DNA Protocol (Thermo Scientific – attached, you can also find this on the webbook or in Lab Files).

Singer *et al*. Characterization of PicoGreen Reagent and Development of a Fluorescence-Based Solution Assay for Double-Stranded DNA Quantitation. Anal Biochem. 1997 Jul 1;249(2):228-38.

Attention:

Read the attached Thermo Scientific protocol because it contains detailed information and cautions.
This protocol is written based on the Thermo Scientific protocol with modifications from Kelli and Anhthu.

Reagents and Materials needed:

1. **Quant-iT** (Invitrogen, P7589) kit contains the following:
   d. **20x TE buffer** stored at room temperature on the shelf for RNA reagents
   e. **PicoGreen reagent** stored in aliquots of **5 µL/tube** in the large DNA **-20°C freezer**. *Caution: PicoGreen is light sensitive!*
   f. **DNA standard** (1 mL Lambda DNA standard (Component C)) stored at 4°C. *Note: PicoGreen reagent can be purchased separately (Invitrogen, P7581).*
2. **Autoclaved DEPC-treated water**
3. Nuclease-free **AMBER brown** or foil-covered 1.5 mL microcentrifuge tubes
4. Nuclease-free pipet tips
5. Pipetman sets (P-2 or P-10, P-100, P-200 and P-1000)
6. 14-mL Nuclease-free centrifuge tube
7. Microcentrifuge tube rack
8. A rack for 14-mL centrifuge tube or a 150-mL glass beaker to hold the tube
10. A box of Kimwipes
11. Vortex mixer
12. Microcentrifuge
13. A Revco cardboard box for holding microfuge tubes (only if clear tubes are used)
14. Aluminum foil for covering the microfuge rack (only if clear tubes are used)

PROCEDURE

A. MEASUREMENT OF EXPERIMENTAL SAMPLES

<u>Note:</u>

- *Standard curve must be generated and saved before the measurement of experimental samples proceeds. If they are not generated, please follow section B. However, if standard curves are already generated, then proceed with the protocol.*
- *The fluorescence of the PicoGreen dye decreases over time, even when protected from light. Process your samples quickly (within 2 hrs).*
- *Nanodrop recommends diluting the UNKNOWN DNA with 1x TE buffer at an estimated DNA concentration that will fall in the middle of the standard curve. However, you do not know the concentration of your DNA samples.* ***What can you do?***

28. Prepare 2 mL of 1x **TE buffer** for **diluting** the **PicoGreen stock** solution (**aliquot of 5 µL**) and **DNA samples** as following:

	1.5 mL	**2 mL**
20X TE buffer stock	75 µL	100 µL
Autoclaved DEPC-treated water	1,425 µL	1,900 µL

    Vortex briefly to mix the contents.
    Spin briefly.

29. Remove **ONE** aliquot (**5 µL)** of **PicoGreen** solution from the Revco cardboard box labeled "**PicoGreen** Stock" in the large **–20°C DNA freezer**. Thaw the tube briefly at room temperature. Flick the tube gently to mix the contents.
    a. Spin briefly.
    b. Add **995 µL of 1x TE buffer** to achieve a 1:200 dilution.
    c. Vortex briefly to mix.
    d. Spin briefly.
    e. Place tube in microfuge tube rack. <u>Note:</u> *If clear tubes with aluminum foil covers are used, use a piece of aluminum foil to cover the microfuge tube rack or put tubes in a Revco box to avoid exposing* ***PicoGreen*** *dye to light. Alternatively, use AMBER tubes.*

30. Label 1.5-mL Nuclease-free **AMBER** microfuge tubes according to the DNA samples and one tube for "Reference." Set tubes on a microfuge tube rack.
31. Pipet **3 µL** of **1x TE buffer** into each of the appropriate amber sample tubes, and **4 µL** of **1x TE buffer** into the **"Reference"** tube.
32. Pipet **1 µL** of **DNA solution** into the appropriate amber sample tubes. Mix by pipetting up and down 5 times. *Note: The total volume is **4 µL**.*
33. Pipet **4 µL** of the **1:200 dilution of PicoGreen** to the "**Reference**" tube. Mix by pipetting up and down for 5 times or flicking the tube to mix the contents.
34. Repeat step 6 for each tube of DNA sample.
35. After you have finished adding diluted **PicoGreen** solution to all DNA samples. Spin tubes briefly in a microfuge. *Note: If you are not using amber tubes, **immediately** place the tube in the Revco cardboard box or on a microfuge tube rack that is covered with a piece of aluminum foil.*
36. **Incubate** the mixtures for **5 minutes** at room temperature. *Note: It is okay to incubate for more than 5 minutes*.
37. Open the arm of the Nanodrop Fluorospectrometer or "Fluorodrop." Clean the pedestals with a piece of Kimwipes wetted with either **freshly prepared** DEPC-treated water (NOT autoclaved) or RNAse-Zap solution. **Blot** (**NOT** swipe) off excess liquid with another piece of Kimwipes.
38. Pipet **1.5 µL** of **autoclaved DEPC-treated water** on the LOWER pedestal. Close the arm so the upper bushing interfaces with the water droplet.
39. Open the arm and blot off the water from both surfaces with a piece of Kimwipes.
40. Open the **ND-3300 software** by clicking on the **ND-3300 icon** on the desktop of the computer --> Click on the **Nucleic Acids** button --> Select **PicoGreen-DNA** from the pull down menu.
41. Pipet **1.5 µL** of **Reference solution** to the LOWER pedestal. Close the arm. Click the **BLANK** button. Blot off the Reference solution from the pedestals. *Note: Nanodrop and Anhthu recommend blanking with 1x TE and then measuring the fluorescence of the Reference as a negative control. If this approach is taken, the RFU reading of the reference should be in the range of 15-45.*

42. Upload the saved **PICOGREEN STANDARD** curve by clicking on **STANDARD CURVES** pull down menu --> Selecting **LOAD** --> Selecting "**PicoGreen Std Curve.nfs**" file from the Standard curves folder --> Click **OK** button. *Note: This standard curve can be used to measure diluted DNA samples with a concentration range of 1* pg/μL to 1,000 pg/μL.
43. Click on the **MEASUREMENT TYPE** box --> Select REFERENCE → Select the desired units (pg/μL)
44. Pipet another **1.5 μL** of **Reference solution** to the LOWER pedestal. Close the arm. Click the **MEASURE** button. *The RFU reading should be 0 if the Reference solution was used as the Blank.*
45. Blot off the solution from the pedestal surfaces with Kimwipes.
46. Repeat reading **Reference** solution for the total of **3-5 times**, each time with a new 1.5 μL solution, NOT reading one droplet for 3-5 times.
47. Click on the **MEASUREMENT TYPE** box --> Select SAMPLES.
48. Enter sample **ID Information**, e.g. Soybean Epidermis.
49. Enter the dilution factor. If you prepared your sample with 1 μL of DNA, 3 μL of 1x TE and 4 μL of diluted **PicoGreen**, then the dilution factor is 8.
50. Pipet **1.5 μL** of **DNA sample solution** to the **lower pedestal**. Close the arm. Click the **MEASURE** button. *Note: Pay attention to a read-out concentration of the DNA sample. This is the concentration of an 8-fold diluted DNA solution.*
51. Blot off the solution from the pedestal surfaces.
52. Repeat reading DNA solution for the total of **3-5 times**, each time with new 1.5 μL solution, NOT reading one droplet for 3-5 times.
53. After all concentration readings for all experimental samples are done, click on the **SHOW REPORT button** --> Select File pull down menu --> Select SAVE WINDOW --> Type in Name of the file in the FILE NAME field --> Click OK button. *Note: You also want to print out the report by Selecting PRINT WINDOW from the File pull down menu. The print out will display the **actual concentration** of your DNA sample, not the 8-fold diluted concentration.*

Attention:

What happens if the standard curve is not appropriate for a certain DNA sample?

*There is a **warning message** saying that **the concentration is out of range**. If the DNA sample is too concentrated, either use the NanoDrop 1000 or dilute the sample with 1x TE and 1:200 diluted PicoGreen. Change the dilution factor.*

***IF** you forgot to report your dilution factor before measuring your samples, then you need to calculate the actual concentration of DNA samples by multiplying the read concentration with the dilution factor (fold of dilution with 1x TE buffer and diluted PicoGreen solution). How? Open the Hard drive C → the ND3300 folder → the Default folder → PicoGreen folder → Select the file of your DNA concentration → Right-click and select OPEN WITH Excel → Copy relevant columns up to the column with Units and all rows with samples → Paste the copied information to a NEW Excel file → Calculate DNA concentration and yield as exemplified below*

Concentration and Yield of DNA Isolated from Soybean Leaves in Paraffin Mixtures

Method: **PicoGreen - DNA**
Software: 2.5.0

Sample ID	Standard Curve	Date	Conc. (pg/uL)	Average Conc. (pg/uL)	Dilution Factor	Calculated Conc. (ng/uL)	Volume of DNA (uL)	Amount of DNA (ng)
Blank	PicoGreen	11/3/06	0					
Reference	PicoGreen		0					
			0					
Soy Leaf1 Paraffin1	PicoGreen		141.28	138.1	4	0.552	20	11.0
			134.87					
Soy Leaf2 Paraffin1	PicoGreen		584.28	585.2	4	2.34	20	46.8
			586.1					

B. PREPARATION OF DNA STANDARD CURVE

Note:

- ***Once the standard curve is created and saved, it does not need to be generated again until a new lot of PicoGreen is used*.**
- *All work is done under normal light conditions in the room. That is, NO light is turned off.*
- *Invitrogen's **PicoGreen** kit comes with a **DNA standard** (1 mL Lambda DNA standard (Component C)) stored at 4°C. However, any clean DNA can be used as the DNA stock. I (Kelli) have used 1 kb DNA ladder from Invitrogen as a DNA stock.*

1. Prepare **2 mL** of **1x TE buffer** from **20x TE buffer** in a 14-mL Nuclease-free tube.

	1.5 mL	**2 mL**
20X TE buffer stock	75 µL	100 µL
Autoclaved DEPC-treated water	1,425 µL	1,900 µL

   Vortex to mix the contents. Spin briefly.

2. Prepare a 10,000 pg/µL solution of stock DNA. Confirm the concentration of DNA on the NanoDrop-1000. Check the A_{260}/A_{280} and A_{260}/A_{230} ratios.
3. Label SEVEN 1.5-mL **AMBER** microfuge tubes D1-D7.
4. Prepare serially diluted solutions at 2x (twice the final) concentrations for standard curves.

DNA standard (1 pg/µL - 1000 pg/µL)

Tube #	Stock DNA Conc. (pg/µL)	Volume of DNA Stock (µL)	Volume of 1x TE (µL)	2x DNA Std Conc. (pg/µL)	Final Conc. In Assay (pg/µL)
D1	10,000	40	160	2,000	1,000
D2	2,000	50	50	1,000	500
D3	1,000	20	80	200	100
D4	200	25	75	50	25
D5	50	40	60	20	10
D6	20	50	50	10	5
D7	10	20	80	2	1

Ref	0	0	50	0	0

<u>Note:</u> *For each diluted solution, vortex briefly and spin briefly.*

- Discard 100 μL of solution in tube #**D1**.
- Discard 30 μL of solution in tube #**D2**.
- Discard 25 μL of solution in tube #**D3**.
- Discard 10 μL of solution in tube #**D4**.
- Discard 30 μL of solution in tube #**D6**.
- Discard 50 μL of solution in tube #**D7**.
- Place tubes on the rack for microfuge tubes.

5. Prepare a **200-fold diluted PicoGreen solution**.
    a. Remove ONE tube of the aliquot **(5 μL)** of **PicoGreen** solution from the -20°C DNA freezer.
    b. Spin briefly.
    c. Add **995 μL** of **1x TE buffer**.
    d. Vortex briefly to mix.
    e. Spin briefly.
6. Prepare the DNA standard curve.
    c. Add **50 μL** of the **1:200 diluted PicoGreen** to each tube containing **50 μL** of the standard solutions. Vortex briefly. Spin briefly.
    d. **Incubate** the mixtures for **5 minutes**. <u>Note:</u> *it is okay to incubate for more than 5 minutes*.
7. Prepare a **REFERENCE** solution as following:

    1x TE buffer 50 μL

    1:200 diluted **PicoGreen** solution 50 μL

    Vortex briefly. Spin briefly. **Incubate** for **5 minutes**. <u>Note:</u> *It is okay to incubate for more than 5 minutes*.
8. Open the **ND-3300 software** on the desktop of the computer --> Click the **Nucleic Acids** button --> Select **PicoGreen - DNA**.

9. Clean the sampling pedestals with a piece of Kimwipes wetted with either the RNAse-ZAP solution or freshly prepared DEPC-treated water.
10. Pipet **2 µL** of **autoclaved DEPC-treated water** on the LOWER pedestal. Close the arm so that upper bushing interfaces with the water droplet.
11. Open the arm and use a Kimwipes to **BLOT away** (NOT wipe off) the water from both pedestal surfaces. *Note: Make sure that the optical surfaces are free of lint before proceeding.*
12. Pipet **2 µL** of **REFERENCE** solution to the LOWER pedestal. Close the arm. Click the **BLANK** button. Blot off the Reference solution from the pedestals. *Note: Nanodrop and Anhthu recommend blanking with 1x TE and then measuring the fluorescence of the Reference as a negative control. If this approach is taken, the RFU reading of the reference should be in the range of 15-45.*
13. Click the **MEASURMENT TYPE** drop box --> Select **REFERENCE** --> Select desired **units (pg/µL)**.
14. Pipet another **2 µL** of the **REFERENCE solution** (0 pg/µL) onto the LOWER pedestal. Close the arm. Click the **MEASURE** button. *The RFU reading should be 0 if the Reference solution was used as the Blank.*
15. Blot off the solution from the pedestal surfaces with Kimwipes.
16. Repeat reading **REFERENCE** solution for the total of **3-5 times**, each time with a new 2 µL of solution, NOT reading one droplet for 3-5 times.
17. Click on the **MEASUREMENT TYPE** drop box --> Select **STANDARD 1**.
18. Enter the **FINAL ASSAY** concentration of **STANDARD1**.
19. Pipet **2 µL** of the **STANDARD 1 solution** onto the LOWER pedestal. Close the arm, and click the **MEASURE** button. Blot off the sampling pedestals.
20. Measure **3-5 replicates** for each diluted PicoGreen standard solutions. *Again, use fresh droplet of solution for each measurement*.
21. Once the standard curve is completed, save the **PICOGREEN** STANDARD curve by selecting **STANDARD CURVES** pull down menu --> Select "**SAVE AS...**"

--> Create a Folder for STANDARD CURVES --> Select that folder --> Type in "**PicoGreen Std Curve_date**" --> Click **OK** button. *Note: The saved standard curve will be loaded during the measurement of experimental samples*.

23. Print out the new standard curve and post on the wall.

At the end of this work, you have a DNA standard curve for determination of concentrations of Experimental DNA samples. You don't need to generate a NEW standard curve until a new lot of PicoGreen is used.

PROTOCOL ND-3300

PicoGreen® Assay for dsDNA

Introduction

The PicoGreen® dye is a fluorescent nucleic acid stain for quantitating double-stranded DNA (dsDNA). Used in conjunction with the micro-volume capability of the Thermo Scientific NanoDrop 3300 Fluorospectrometer, the PicoGreen® assay provides a highly sensitive means of dsDNA quantitation with minimal consumption of sample. The main disadvantage of general UV spectroscopy for dsDNA quantitation is the contribution of signal from single-stranded DNA (ssDNA) and other contaminants, such as protein and extraction buffers. PicoGreen® reagent circumvents such contributions from interfering substances by exhibiting an emission maximum at 530nm when bound specifically to dsDNA (unbound PicoGreen® reagent exhibits minimal fluorescence in solution). The ability of the NanoDrop 3300 to measure as little as 1 ul of sample, allows significantly scaled-down reaction volumes, thereby using only a fraction of sample commonly needed for conventional cuvette-based fluorometers. The NanoDrop 3300 has demonstrated a detection range for dsDNA bound with PicoGreen® reagent of 1 ng/ml – 1000 ng/ml, and has proven to be 75 times more sensitive than using the Hoechst 33258 dye with this system. Readings taken at the lowest detection limit consume only 2 picograms of dsDNA per measurement. The following protocol is an adaptation of the Molecular Probes™ dsDNA Quantitation Reagent and Kits product information sheet.

PicoGreen® Assay Supplies

Equipment:

- NanoDrop 3300 Fluorospectrometer
- 2uL pipettor (low retention nuclease free pipette tips)

Materials:

- Low lint laboratory wipes
- Nuclease free amber or foil covered 1.5 ml polypropylene tubes

Reagents:

- PicoGreen® dsDNA reagent kit (Molecular Probes catalog # P-11496)
- Nuclease free dH_2O

PicoGreen® Assay Protocol

This protocol is written to accommodate small volume reactions (10 ul total volume per tube.) ranging from 10 ul and 200 ul total reaction volumes and have been shown to yield comparable results.

1. Prepare 1X TE with nuclease free dH_2O. The volume needed depends on total number of samples to be measured and the volume of PicoGreen® working solution needed.
2. Dilute the concentrated PicoGreen® Dye stock two-hundred (200) fold.

   Example: For the PicoGreen® working solution, dilute the PicoGreen® stock (200X concentrate) by mixing the dye thoroughly and transferring 995 ul of 1X TE and 5ul of the dye stock to a 1.5 ml amber snap cap tube. Mix well and protect from light. *(Prepare fresh as the diluted PicoGreen® dye is stable for only a few hours).*
3. Thaw the standard and unknown dsDNA samples. Once thawed, mix each individual solution gently but thoroughly.
4. Prepare serially diluted dsDNA standards at 2X the final concentrations in nuclease free vials or tubes (please refer to the example standard curve dilution series on page 2).
5. Transfer one volume of the diluted dsDNA standards into labeled nuclease free amber or foil covered tubes.
6. Aliquot an equal volume of diluted unknown dsDNA samples into appropriately labeled nuclease free amber tubes.

   Note: It is recommended that dilutions of unknown dsDNA samples be performed in 1X TE at an estimated concentration that will likely fall within the standard curve.
7. Transfer an equal volume of the PicoGreen® working solution to each amber tube containing either the 2X standard dsDNA solution or unknown sample.
8. Prepare the or Reference solution (negative control) by adding equal volumes of 1X TE and PicoGreen® working solution.
9. Mix each standard dilution and unknown sample thoroughly and allow to equilibrate at room temperature for five minutes.
10. Proceed to the NanoDrop 3300 standard curve protocol.

Standard Curve Protocol

1. Clean both sampling pedestals with 2 uL of nuclease free deionized water.
2. Open upper arm and firmly blot the two pedestals with a dry lab wipe. Make sure there are no traces of lint on the pedestals before continuing.
3. Open the operating software. Click on the Nucleic Acid Quantitation button and select the PicoGreen method.
4. Add 2 uL of assay buffer (no dye, no sample) to the lower pedestal. Lower the arm and click F3 or the Blank button. When the measurement is complete, lift the arm and use a dry laboratory wipe to blot the buffer from both the bottom and upper measurement surfaces. Use a fresh aliquot of buffer to verify a proper baseline.
5. Under Measurement type, click on the Standards tab. Highlight the Reference standard.
6. Mix the reference solution (assay buffer and dye, no sample) briefly and transfer 2 uL of the solution onto the lower pedestal. Lower the arm and click F1 or the Measure button. A pop up window will ask for confirmation of the units. (Recommended ng/mL or pg/uL)
7. Measure up to 5 replicates of the reference solution using a fresh 2 uL aliquot for each measurement.
8. Select Standard 1 to enter a value. Enter values for up to 7 standards.
9. Mix the standard solution briefly and transfer 2 uL onto the lower pedestal. Lower the arm and click F1 or the Measure button. Measure up to 5 replicates of each standard using a fresh 2 uL aliquot for each measurement.
10. Once the standard curve is completed, select the Standard Curve Type (Interpolation, Linear, 2° polynomial, 3° polynomial) that best fits the standards data set.
11. Click on the Sample tab under Measurement Type, and enter the unknown samples' respective ID information. If a dilution of the unknown sample was made, enter the dilution factor in the box below the sample ID window.
12. Add 2 ul of the sample and use the F1 key or click the Measure button to initiate the measurement cycle. Use a fresh aliquot of sample for each measurement.

Example of standard curve dilution series.

Stock dsDNA (ng/ml	2X dsDNA volume (ul)	1X TE buffer volume (ul)	2X dsDNA standard (ng/ml)	Final dsDNA (ng/ml)
2000	10*	0	2000	**1000**
2000	10**	10	1000	**500**
1000	4	16	200	**100**
200	5	15	50	**25**
50	8	12	20	**10**
20	10	10	10	**5**
10	4	16	2	**1**
0	0	10	0	**0**

Initial stock undiluted* * Initial stock diluted 1:1*

- The final concentration of the 1:1 dilution of PicoGreen® working solution is shown in the last column the table 1.
- The software will generate standard curves using between 1 to 7 standard concentrations along with the reference solution.
- When determining dsDNA concentrations near the lower detection limit, use a higher density of standards at the lower end.
- Excess volume of the 2x standards are prepared to facilitate the easy transfer of 5 ul into amber tubes during step 7 on page 1.

Example spectrum of a PicoGreen-dsDNA sample