Non Controlled Version

**Note: Controlled Versions of this document are subjected to change without notice*

Strand Specific 96 Well cDNA Synthesis

I. Purpose

To synthesize, in a 96-well format, cDNA for strand specific RNA-Seq (WTSS) Illumina library construction

II. Scope

All procedures are applicable to the BCGSC Library Core group and the Library Technology Development group

III. Policy

This procedure will be controlled under the policies of the Genome Sciences Centre, as outlined in the Genome Sciences Centre High Throughput Production Quality Manual (QM.0001). Do not copy or alter this document. To obtain a copy see a QA associate

IV. Responsibility

It is the responsibility of all personnel performing this procedure to follow the current protocol. It is the responsibility of the Group Leader to ensure personnel are trained in all aspects of this protocol. It is the responsibility of Quality Assurance Management to audit this procedure for compliance and maintain control of this procedure.

V. References

Reference Title	Reference Number
N/A	N/A

VI. Related Documents

Document Title	Document Number
Operation and Maintenance of the Agilent 2100 Bioanalyzer for DNA samples	LIBPR.0017
96-well DNA Purification Using Ampure (or XP Beads) Magnetic Beads and Biomek FX	LIBPR.0047
Manual Bead Clean using Ampure XP Beads	LIBPR.0073
Operation and Maintenance of the Agilent 2100 Bioanalyzer for RNA samples	LIBPR.0018

VII. Safety

All Laboratory Safety procedures will be complied with during this procedure. The required personal protective equipment includes a laboratory coat and gloves. See the material safety data sheets (MSDS) for additional information.

VIII. Materials and Equipment

Name	Supplier	Number: #	Model or Catalogue #	
Fisherbrand Textured Nitrile gloves – various sizes	Fisher	270-058-53		✓
RNAse Zap	Ambion	9780		✓
Ice bucket – Green	Fisher	11-676-36		✓
wet ice	In house	N/A	N/A	N/A
RNAse free 1.5 ml eppendorf tube	Ambion	12400		✓
Gilson P2 pipetman	Mandel	GF-44801		✓
Gilson P10 pipetman	Mandel	GF-44802		✓
Gilson P20 pipetman	Mandel	GF23600		✓
Gilson P200 pipetman	Mandel	GF-23601		✓
Gilson P1000 pipetman	Mandel	GF-23602		✓
Diamond Filter tips DFL10	Mandel Scientific	GF-F171203		✓
Diamond Filter tips DF30	Mandel Scientific	GF-F171303		✓
Diamond Filter tips DF200	Mandel Scientific	GF-F171403		✓
Diamond Filter tips DF1000	Mandel Scientific	GF-F171703		✓
VX-100 Vortex Mixer	Rose Scientific	S-0100		✓
Large Kimwipes	Fisher	06-666-117		✓
Black ink permanent marker pen	VWR	52877-310		✓
Bench Coat (Bench Protection Paper)	Fisher	12-007-186		✓
Small Autoclave waste bags 10"X15"	Fisher	01-826-4		✓
DEPC water	Ambion	9922		✓
Mussel Glycogen	Roche Diagnostics	901393		✓
100% Ethanol	Commercial	NA	NA	NA
3M Sodium Acetate, pH 5.5	Invitrogen	9740		✓
70% Ethanol	Made in house		NA	NA
Mini-centrifuge	Eppendorf	5417R		✓
Rainin RNase free barrier tips	Rainin	RT-L10F		✓
Pipet-Lite™ XLS P2-20 12 channel	Rainin	L12-20XLS		✓
Peltier Thermal Cycler	MJ Research	PTC-225		✓
Plastic seal (3M)	Qiagen	19570		✓
MicroAmp Clear Adehesive Film (PCR seal)	Applied Biosystems	4306311		
5X first strand buffer	Invitrogen	from kit #18090-019		

RnaseOUT (40U/µL)	Invitrogen	from kit #18090-019		
10mM dNTP mix	Invitrogen	from kit #18090-019		
SS II RT	Invitrogen	from kit #18090-019		
5x Second strand buffer	Invitrogen	from kit #18090-019		
dNTP Blend, 12.5 mM with dUTP, 1mL	GeneAmp	N8080270		✓
100mM DTT	Invitrogen	from kit #18090-019		
E.Coli DNA Ligase (10U/µL)	Invitrogen	from kit #18090-019		
E.Coli DNA Polymerase (10U/µL)	Invitrogen	from kit #18090-019		
E.Coli RNAseH (2U/µL)	Invitrogen	from kit #18090-019		
DEPC water	Ambion	from kit #18090-019		
Random hexamer primer, 50 ng/µl (25uM)	Invitrogen	from kit #18090-019		
Actinomycin D 5mg	Affymetrix	10415		

IX. Procedure

1. General guidelines and input material QC:

1.1. The input material for this pipeline is polyA+ RNA (messenger RNA) fraction which has been on-column DNAse I treated, in a 96-well AB1000 plate.

1.1.1. Prior to starting this protocol 1µL of polyA+ fraction should be assessed for both quality and quantity via Agilent mRNA Nano Assay using 1/5 dilution of the marker. For all samples Agilent mRNA concentration (1/5 dilution of the marker) should not be below 15ng/µL. Refer to LIBPR.0018 Operation and Maintenance of the Agilent 2100 Bioanalyzer for RNA samples.

1.1.2. In order to obtain good quality data the recommended minimum input amount per sample as per Agilent mRNA Nano Assay (1/5 dilution of marker using 1.5µL (out of 10µL total) going into denaturation) is:

15ng/µL* 8µL (volume moving forward) = 120ng. (1/5 marker dilution)

120ng/5 = approximately 20-25ng of mRNA***

Note: ***Poly(A)+ RNA or mRNA makes up between 1-5% of total RNA. The mRNA amount listed above can be obtained from 2ug of total RNA. Using lower than 2µg Total RNA starting amount (and thus subsequently lower mRNA starting amount) has to be done with the understanding that it may result in a lower quality of data or even, in some cases, in library construction failure.

1.2. The input volume for this protocol is 8µL/well in SuperaseIn treated DEPC water (1µL SuperaseIN for every 20µL of DEPC water).

1.3. Reactions should never be vortexed and plate covers are never to be re-used.

1.4. P200 manual pipette is more accurate than P1000, therefore whenever possible use a P200 for brew preparation.

1.5. The working stock of Actinomycin D should be prepared fresh just before 1st strand synthesis brew set up. Unused Actinomycin D should be discarded.

1.6. Enzymes should be taken out of the freezer only just before addition to the brew or sample.

2. First strand cDNA synthesis:

2.1. Wipe down the workbench, small equipment, and ice bucket first with RNAse Zap (Ambion), second with 70% EtOH, and finally with DEPC-water. Lay down new bench coat.

2.2. Retrieve Invitrogen reagents: 5X First Strand Buffer, 100mM DTT, 10mM dNTP mix, and thaw them at room temperature. Once thawed keep reagents on ice.

2.3. Retrieve Invitrogen Random Hexamers (6mer, 50ng/µL) and Actinomycin D (10µg/µL) and place them **on ice**. Actinomycin D is kept in amber tubes as it is light sensitive.

2.4. While the reagents are thawing, use a hand held scanner to generate 1st strand cDNA synthesis brew mix barcode:

2.4.1. LIMS: Using a handheld scanner, enter your name and password, select sequence database, Lib Construction, 5th floor printers, and click "log in" button.

2.4.2. Select Brew Calculator "**Strand specific_cDNA_1st _strand**".

2.4.2.1. If processing a full plate select 96 samples in the second box.

2.4.2.2. If processing a partial plate select the number of wells in the first box and "1" for the second box.

2.4.3. Click "**Mix Standard Solution**" button. See image below:

Figure 1: Mix Standard Solution

2.4.4. Under parameters, if processing more than one plate enter the number of plates. If processing less than a full plate leave "1" for plates and enter number of wells. Click "Re-calculate Standard Solution" red button. See image below:

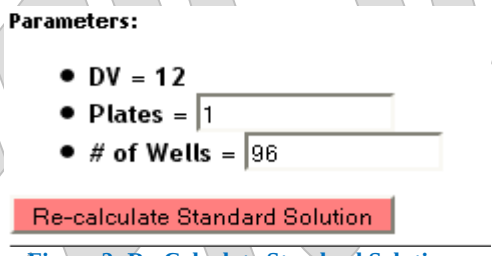


Figure 2: Re-Calculate Standard Solution

2.4.5. Scan the reagent barcodes into their appropriate boxes.

2.4.6. In Other Parameters section, select Type: "Reagent", Grp: "Lib_Construction", Barcode Label: "1D Large Solution/Box/Kit Labels".

2.4.7. Go over your entries and check if everything is correct. Click on "Save Standard Mixture" red button (see image above).

2.4.8. Retrieve 1D Large label from 5th floor printer outside the RNA room and brew mix check-list label from RNA Room printer.

2.5. If LIMS is down, use the Strand Specific First strand cDNA synthesis brew calculator spreadsheet to make up the brew. Print the spreadsheet and save it in your lab book. The excel file is located in R:\Lib core\Work Sheets and Calculators\Plate Based\Strand Specific and is called "Strand specific_96well_First strand cDNA synthesis_130528nd.xlsx". See Appendix B.

2.6. Vortex and pulse spin Random Hexamers (6mer, 50ng/µL), then

2.6.1. If processing a full plate, aliquot 40µL/well of Random Hexamers into one row (12 wells) of a 96-well plate to be used as a reservoir. See Section 1 of Strand Specific First strand cDNA synthesis calculator (Appendix B).

2.6.2. If processing less than one plate, aliquot the appropriate amount of random hexamers (based on the number of rows) plus dead volume into one row of 96-well plate (example: for 3 rows of samples this will be: (4µL*3) + 5µL = 17µL/well).

2.7. Cover the plate with plastic cover and spin down at 4°C, 2000g for 1 min.

2.8. From -80°C freezer retrieve the plate containing previously QCed polyA+ RNA fraction (8.0µL/well). Thaw it on ice. Spin down the plate at 4°C, 2000g for 1min. Place it back on ice.

2.9. Using Rainin Manual multichannel P2-20 pipette, add 4µL of Random Hexamers into each well (row by row). Check the volume in each tip. Pipette each row up and down 5 times to mix. While mixing, watch the volume in each tip to make sure that all wells within a row are getting mixed. Change tips between rows. Place a checkmark on the side of each row after each random hexamer addition to make sure that no rows are skipped or repeated.

2.10. Cover the plate with plastic seal. Spin down the plate at 4°C, 2000g for 1min. Place it on ice.

2.11. Remove from ice and thaw Actinomycin D solution (10µg/µL).

2.12. Once thawed, make 1/10 dilution of Actinomycin D with DEPC water in a fresh 1.5mL amber tube:

2.12.1. For each plate mix 13uL of Actinomycin D (10µg/µL) with 117 µL of DEPC water (1/10 dilution to a final, working concentration of 1µg/µL).

2.12.2. Mix well by gentle, repeated, pulse-vortexing, and spin down in a minifuge. Protect it from light and keep it on ice.

Note: *Actinomycin D working stock solution (1µg/µL) must always be prepared fresh – just before you are ready to use it. Once thawed, any left over Actinomycin D (stock or diluted) should be discarded into the designated waste container.*

2.13. Prepare Strand specific 1st strand cDNA synthesis brew following the printed LIMS calculator. If LIMS is down, prepare Strand Specific First strand cDNA synthesis brew following the printed Excel spreadsheet. section 2 (See Appendix B). Make sure to mix each reagent well before adding to the brew. Add enzymes last.

2.14. Mix the brew very well by *gentle* repeated pulse-vortexing. Keep the brew on ice. Discard unused Actinomycin D into its designated waste containter.

2.15. For one full plate, aliquot 96µL of the above brew into one row (12 wells) of a 96-well plate. **Do not throw away left over First Strand brew.**

2.16. When processing a partial plate, aliquot appropriate amount of the brew (based on the number of rows) plus dead volume, into one row of a 96-well plate (for example: for 3 rows of samples this will be (11µL*3) +5 µL = 38µL /well. The minimum dead volume is 5µL but when processing more than 5 rows the dead volume should be set to the number of rows (for 6 rows = 6µL).

2.17. Seal the plate with plastic cover and spin down at 4°C, 2000g for 1min. Place it on ice.

2.18. Heat denature poly (A) + RNA fraction with random hexamers at 70°C for 5 min in the tetrad termocycler.

2.19. After 5 min denaturation, chill the plate on ice for 1min. Spin down the plate at 4°C, 2000g for 1min and place it back on ice.

2.20. Using Rainin manual multichannel P2-20 pipette add 11µL of the brew into each well (row by row). Pipette each row up and down 5 times to mix. While mixing, watch the volume in each tip to make sure that all wells within the row are being mixed. Change tips between rows. Place a check mark on the side of each row after brew addition to make sure that no rows are skipped or repeated.

2.21. Seal the plate with plastic cover and spin down at 4°C, 2000g for 1min.

2.22. Incubate the reaction plate at room temperature (21-22°C) for 10min.

2.23. While the plate is incubating, retrieve Invitrogen Superscript II RT (200U/µL) from the -20°C freezer. Make up diluted SuperScript II RT mix following section 3 of the Strand Specific First Strand cDNA synthesis worksheet. Mix the tube gently few times by flicking and pulse spin. Place on ice and label as diluted SSIIRT.

2.24. On ice,

2.24.1. for one full plate aliquot 28.2 µL of the diluted SSIIRT enzyme into each well of one row, 12 wells of a 96-well plate.

2.24.2. If processing a partial plate, aliquot appropriate amount of diluted SSIIRT (based on the number of rows) plus dead volume into one row of 96-well plate. For example, for 3 rows of samples this will be: (2µL*3) + 5µL= 11µL/well) When processing more than 5 rows the dead volume should be set to the number of rows.

2.25. Spin down the plate at 4°C, 2000g for 1min. Place it back on ice.

2.26. After the 10min room temperature incubation use manual Rainin multichannel P2-20 pipette to add 2µL of diluted SSIIRT to each well (row by row). After each addition, mix 5 times. Change tips between each row. To assure that no rows are skipped or repeated, place a check mark on the side of each row after enzyme addition.

2.27. Change the setting of the Multichannel pipette to 15uL and using new tips pipette each row up and down 5 times to mix. While mixing, monitor the volume in each tip to make sure that all wells within a row are being mixed. Change tips after each row. Mark each row with a checkmark to make sure that no rows are skipped.

2.28. Seal the plate well with PCR cover. Spin down at 4°C, 2000g for 1min.

2.29. In the tetrad thermocycler incubate the 1st strand cDNA synthesis reaction at 45°C for 1h. Use tetrad program cDNA1 in the ssWTSS folder.

2.30. Retrieve Ampure XP beads from their 4°C storage location on the 5th floor. Mix the beads very well by inverting until the solution becomes uniform.

2.30.1. For processing a full plate, transfer 45mL of XP beads into a fresh 50mL Falcon tube.

2.30.2. When processing a partial plate, remove the appropriate volume of beads depending on the number of rows being processed. For each row you will need 1.4mL of beads solution (100µL/well plus dead volume).

2.30.3. Leave the aliquoted XP beads at room temperature. Place the stock bottle back into its designated 4°C storage location.

2.31. Retrieve the non-enzymatic reagents needed for second strand cDNA synthesis: 5X Second Strand buffer and 12.5mM GeneAmp dUTP mix. Thaw them at RT for few minutes and once thawed immediately transfer to ice. Leave enzymes in the freezer.

2.32. After the tetrad program 1h incubation is over, remove the plate from the thermo cycler, spin it down at 2000g, for 1min, and place the plate on ice.

3. DEPC water addition

3.1. If processing full plates only, the Biomek FX-5, located on the 5th floor, can be used for this step. If processing a partial plate, a manual multichannel P200 pipette can be used to add DEPC water (see step 3.5 below).

3.2. Decontaminate the Biomek deck with RNAse Zap, then with 70% EtOH, and finally with DEPC-water.

3.3. Log in using your username and password. Open Biomek software, select project "FG Indexing", open program "P165B_384Axygen_to_AB1000".

3.4. Use P165 barrier tips to add 25µL of **DEPC water** to each well. Follow the displayed deck layout for set up and click "OK". When the program is complete, seal the plate with clear tape sealer and place on ice.

3.5. If processing less than one plate, aliquot the appropriate amount of DEPC water, based on the number of rows, into one row of AB1000 plate. Example for two rows: (25µL*2) + 5µL = 55µL. Use manual Rainin multichannel P200 pipette to add 25µL of DEPC water to each row. Mix each row 5 times. Seal the plate and place it on ice.

3.6. Total volume per well should now be 50µL.

4. Ampure XP bead clean up of RNA/DNA hybrid.

4.1. Double check that the total volume per well is 50µL before starting XP clean up procedure.

4.2. **Before use, ensure that the XP beads have completely reached room temperature.** Cold beads will result in reduced recovery.

4.2.1. **For processing of full plates, follow the protocol: LIBPR.0047 – *96-well* *DNA Purification Using Ampure (or XP Beads) Magnetic Beads and Biomek FX*.**

4.2.2. For partial plates, both the protocols: LIBPR.0047 – *96-well DNA Purification Using Ampure (or XP Beads) Magnetic Beads and Biomek FX* or LIBPR.0073 – *Manual Bead Clean using Ampure XP Beads* can also be used in this step (with the same modifications as specified in this section).

4.3. Perform the procedure with the following modifications:

4.3.1. For partial plates, use manual Multichannel P200 to add 100µL of room temperature XP beads into each row and mix 10 times.

4.3.2. For full plates, use the Biomek to add 100µL of XP beads to each well.

4.3.3. Use 10 min XP beads binding time before placing the plate on the magnet.

4.3.4. It is critical to let the beads separate on the magnet for a full 5 minutes. Removing the binding buffer before the beads have completely separated will impact cDNA yields.

4.3.5. For washes, use 70% EtOH prepared specifically for RNA use.

4.3.6. After adding elution buffer, let the plate incubate for 2 min before placing it on the magnet.

4.4. After elution, keep the plate on ice while preparing for the next step. Each well should contain 35µL.

5. Strand Specific Second strand cDNA synthesis.

5.1. LIMS reaction set up:

5.1.1. Log into LIMS. Click on the "Solution" tab. From "Mix Standard Solution" protocol menu select "**Strand Specific_cDNA_2nd strand**".

5.1.1.1. If processing a full plate select 96 samples in the second box.

5.1.1.2. If processing a partial plate select number of wells in the first box and "1" for the second box. Click "**Mix Standard Solution**" button. When processing a full plate:

Figure 3: Mix Standard Solution

5.1.2. Under parameters, if processing more than one plate enter the number of plates. If processing a partial plate leave "1" for plates and enter the number of wells. Click "Re-calculate Standard Solution" red button.

5.1.3. Scan in reagent barcodes.

5.1.4. In Other Parameters section, select Type: "Reagent", Grp: "Lib Construction", Barcode Label: "1D Large Solution/Box/Kit Labels".

5.1.5. Go over your entries and check if everything is correct. Click on "Save Standard Mixture".

5.1.6. Retrieve Brew barcode and Brew check list labels from 5th floor printers inside and outside the RNA room.

5.2. If LIMS is down, use the Strand Specific second strand cDNA synthesis brew calculator spreadsheet to make up the brew. Print the spreadsheet and save it in your lab book. The excel file is located in R:\Lib core\Work Sheets and Calculators\Plate Based\Strand Specific and is called "Strand_specific_96-well_Second strand cDNA synthesis_130528nd.xlsx". See Appendix C

5.3. Prepare Strand specific 2nd strand cDNA synthesis brew following the printed LIMS calculator. If LIMS is down, prepare Strand Specific Second strand cDNA synthesis brew following the printed Excel spreadsheet. See Appendix C. Make sure to mix each reagent well before adding to the brew. Add enzymes last.

5.4. Mix the brew very well by *gentle* repeated pulse-vortexing. Keep the brew on ice.

5.5. For a full plate, aliquot 133uL of the above brew into one row of AB-1000 plate.

5.6. When processing a partial plate, aliquot the appropriate amount of the above brew (based on the number of rows) plus dead volume into one row of AB1000 plate. For example, for two rows of samples this will be, (15µL*2) + 5µL = 35µL/well). When processing more than 5 rows the dead volume (in µL) should equal the number of rows.

5.7. Seal the plate and spin down at 4°C, 2000g, for 1min. Place it on ice.

5.8. Using manual P2-20 Rainin multichannel pipette and Rainin barrier tips, add 15µL of the brew into each well containing XP bead cleaned cDNA. Pipette each row up and down 5 times to mix. While mixing, watch the volume in each tip to make sure that all wells within the row are being mixed. Use new tips for each row. Place a check mark on the side of each row to make sure no rows are skipped or repeated.

5.9. Seal the plate with PCR cover and spin down at 4°C, 2000g for 1min.

5.10. In the tetrad thermocycler incubate the plate at 16°C for 2h 15min. Use tetrad program cDNA2 in the ssWTSS folder.

5.11. This is a safe stopping point. If performing XP bead clean up on the same day, continue to next step.

5.12. Retrieve Ampure XP beads from their 4°C storage location on the 5th floor. Mix the beads very well by inverting until the solution becomes uniform.

5.12.1. For processing a full plate, transfer 45mL of XP beads into a fresh 50mL Falcon tube.

5.12.2. When processing a partial plate, remove the appropriate volume of beads depending on the number of rows being processed. For each row you will need 1.4mL of beads solution (100µL/well plus dead volume).

5.13. Leave the aliquoted XP beads at room temperature. Place the stock bottle back into its designated 4°C storage location.

5.14. After thermocycler program is finished, remove the plate and spin it at 4°C, 2000g for 1min.

5.15. Proceed to step 6.0 below if performing XP bead clean up on the same day. Alternatively, store the plate at -20°C.

5.16. Please complete the relevent steps in the "B-Strand Specific cDNA Synthesis" LIMS protocol. See Appendix D.

6. Ampure XP bead clean up of ds strand specific cDNA:

6.1. Remove beads from 4°C and leave at room temperature for **at least** 15 minutes. **Before use, ensure that they have completely reached room temperature.** Cold beads will result in reduced recovery.

6.2. For processing of full plates follow the protocol: LIBPR.0047 – 96-well DNA Purification Using Ampure (or XP Beads) Magnetic Beads and Biomek FX. Use Biomek FX 5 to add 100µL of room temperature XP beads to each well.

6.3. For partial plates, both the protocols: LIBPR.0047 – *96-well DNA Purification Using Ampure (or XP Beads) Magnetic Beads and Biomek FX* or LIBPR.0073 – *Manual Bead Clean using Ampure XP Beads* can also be used in this step (with the same modifications as specified in this section). Use manual multichannel Rainin P200 to add 100µL of room temperature XP beads to each row. Mix each row 10X.

6.4. Perform the procedure with the following modifications:
6.4.1. Use 10 min XP beads binding time before placing the plate on the magnet.

6.4.2. It is critical to let the beads separate on the magnet for a full 5 minutes. Removing the binding buffer before the beads have completely separated will impact cDNA yields.

6.4.3. For washes, use 70% EtOH prepared specifically for RNA use.

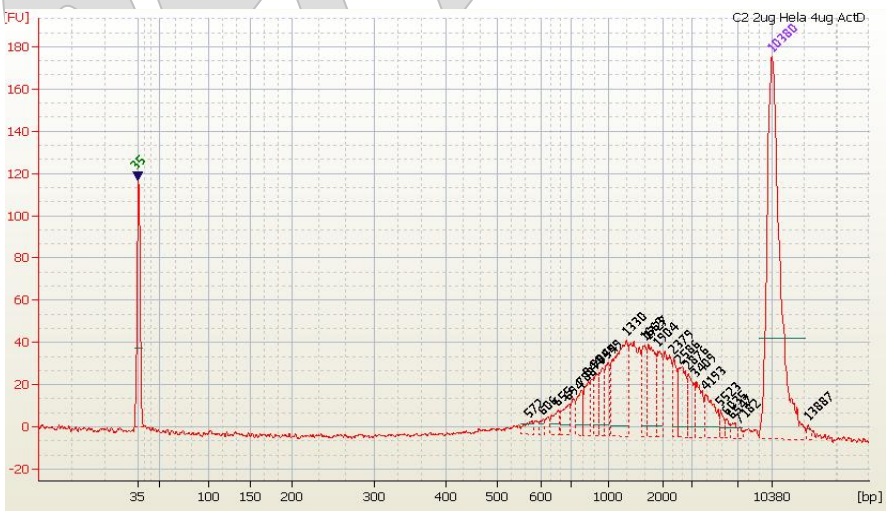
6.5. After elution you can store the plate at -20°C. This is a safe stopping point. If time permits, proceed to the next step.

7. Strand Specific cDNA QC using HS Agilent assay

7.1. For quality and quantity control check of the generated strand specific double stranded cDNA, choose 11 random samples from each plate include the negative and positive controls for HS Agilent assay. A minimum of 2 HS chips should be run for each full plate. For partial plates, your supervisor will let you knowof the wells to QC on HS Agilent.

7.2. Refer to the document: LIBPR.0017 – Operation and Maintenance of the Agilent 2100 Bioanalyzer for DNA samples.

7.3. Example of expected profile for 2ug UHR total RNA starting amount is shown below:



Strand Specific cDNA HS Agilent profile for 2ug Helas3 starting material

7.4. Show your results to your suprervisor.

7.5. Store the plate at -20°C.

7.6. Please complete the relevent steps in the "B-Strand Specific cDNA Synthesis" LIMS protocol. See Appendix D

Appendix A - Actinomycin D

1. Actinomycin D is toxic and gloves must be worn when handling it.

2. Actinomycin D powder is hygroscopic and sensitive to light. When stored in original vial, manufacture sealed and protected from light and moisture, at 4°C, it remains unchanged for the amount of time specified by the manufacture (it is shown on the label). When receiving Actinomycin D powder in LIMS enter that date as the expiration date of the powder.

3. Dilute solutions of Actinomycin D are very sensitive to light. This product tends to adsorb to plastic and glass on standing in solution. It is important to take the aliquots out of the freezer only shortly before you are ready to use it, and after thawing using ActD solution quickly. Once taken out from the freezer, an aliquot should either be used up or discarded – it should never be re-frozen for later use.

4. The shelf life of re-suspended Actinomycin D when kept at 4°C and above is only few hours. It is therefore important to perform the re-suspension procedure in an efficient and quick manner.

5. Protected from light, frozen aliquots of 10µg/µL (or 10mg/mL which is 8mM) are expected to be stable up to 3 months at -20°C. After 3 months, all unused tubes of re-suspended, frozen Actinomycin D should be discarded and a new re-suspension batch should be made.

6. Preparation of 10µg/µL (or 10mg/mL) storage stock of Actinomycin D.

   6.1. Log into LIMS. Go to "Solutions" page. Under "Mix Standard Solutions" select **Actinomycin 10µg/µL** and 1 x 1 for samples (see image below). Click "Mix Standard Solution" button.

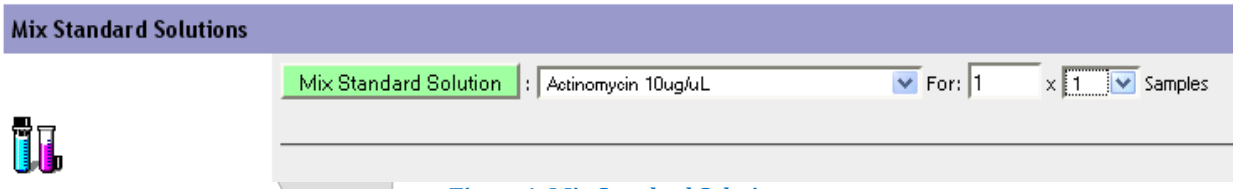


Figure 4: Mix Standard Solution

   6.2. In the Parameters section enter the mg of Actinomycin D in the vial (see image below). Click "Re-calculate Standard Solution".

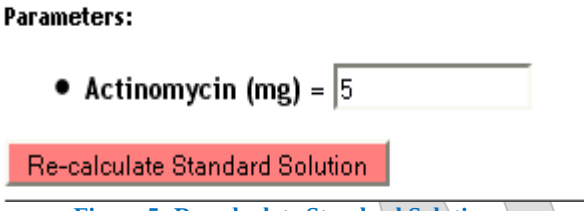


Figure 5: Re-calculate Standard Solution

6.3. In the Other Parameters section enter "Reagent" for Type, expiration date for 3 months ahead, select "Lib Construction" group, 2D solution labels. Click on the "Save Standard Mixture" button (see image below):

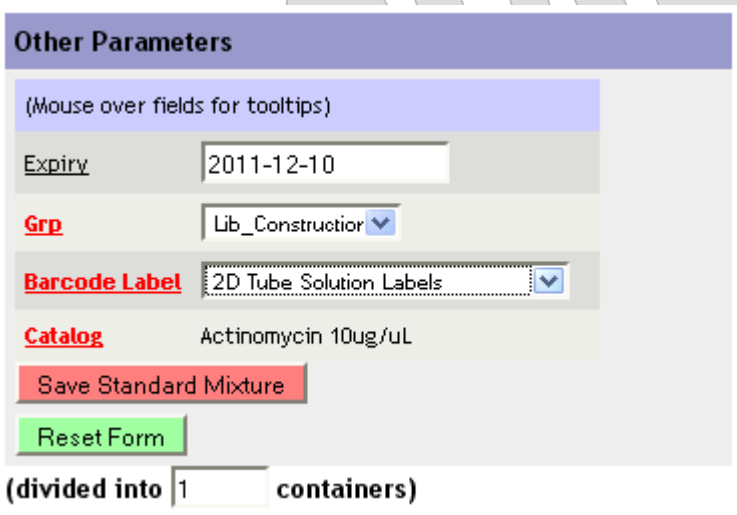


Figure 6: Save Standard Mixture

6.4. Retrieve both the solution barcode and the calculator from the corresponding printers.

6.5. Using the newly generated barcode perform an aliquot step in LIMS dividing the total volume into 15µL per tube. Select 2D solution barcodes.

7. Add the amount of room temperature DMSO listed on the large LIMS calculator to the amber vial containing 5mg of Actinomycin D powder. Close the vial.

8. Mix well few times by gentle pulse-vortexing. Make sure that the powder is fully re-suspended.

9. Open the vial and mix by pipetting using P200.

10. Using 1.5mL amber tubes immediately aliquot Actinomycin D solution 15µL per tube. Place the tubes on ice.

11. Attach 2D solution barcodes generated in step 6.5 on the tubes.

12. Cover each tube with parafilm creating tight seal.

13. As soon as possible place tubes in 9x9 box, protected from light, in the -20°C freezer.

Appendix B: Strand Specific First Strand cDNA Synthesis Brew Calculator

Strand Specific First Strand cDNA Synthesis

Project name: _____ **plate ID: tra** **Date:**_____
Library:
Plate set :

1 Adding Hexamers

poly A+ fraction volume: 8 uL
random hexamers (50ng/uL): _____ 4 uL **Aliquot** **40 uL of the hexamer into one row (12 wells) of a plate.**

Heat denature: 70°C for 5min
Chill on ice for 1 min
Spin down at 4°C for 1min

2 First Strand cDNA synthesis brew (2.27x mix)

1. # of samples **96** editable. If editing # of samples also edit # of rows
2. # of plates **1** editable
3. # of wells for Dead vol. **15.05**
4. # of BrewSourceWells **12**
5. # of rows **8** editable. If editing # of rows also edit # of samples

	Sol #	ul / well	Samples Brew	DV Brew	Total volume brew	( )	Check off each
5X First Strand Buffer		**5**	480	75	555.3	( )	5X First strand buffer
RNaseOut (40U/uL)		**0.6**	57.6	9	66.6	( )	RNaseOut (40U/uL)
10mM dNTP mix		**1.25**	120	19	138.8	( )	10mM dNTP
0.1 M DTT		**2.5**	240	38	277.6	( )	0.1 M DTT
ActinomycinD (1µg/uL)		**1**	96	15	111.1	( )	ActinomycinD (1µg/uL)
DEPC water		**0.6**	57.6	9	66.6	( )	DEPC water
Total volume (ul)		**11.0**	1051	165	1216.0		ul Brew Mix in tube
	Aliquot	**96**	**uL of the brew above into one row (12 wells) of a plate**				

3 Diluted SuperScript II RT

1. # of samples **96** editable. If editing # of samples also edit # of rows
2. # of plates **1** editable
3. # of wells for Dead vol. **73.2**
4. # of BrewSourceWells **12**
5. # of rows **8** editable. If editing # of rows also edit # of samples

	Sol #	ul / well	Samples Brew	DV Brew	Total volume brew	( )	Check off each
2.27X First Strand mix		**0.881**	84.576	64	**149**	( )	2.27X First Strand mix **(leftover from section 2)**
DEPC H20		**0.619**	59.424	45	**105**	( )	DEPC H20
Superscript II RT (200U/uL)		**0.5**	48	37	**85**	( )	Superscript II
Total volume (ul)		**2.0**	192	146	**338**		
	Aliquot	**28.2**	**uL of the brew above into one row (12 wells) of a plate**				

4 Manually add SuperScript II Reverse Transcpriptase

Diluted Superscript II RT **2** uL per well

total 1st strand cDNA synthesis reaction volume : **25uL**

Appendix C: Strand Specific Second Strand cDNA Synthesis Brew Calculator

Strand Specific: Second Strand cDNA synthesis brew:

volume in plate after XP bead clean up 35uL

Project name: _____ **plate ID: tra** **Date:**_____
Library:
Plate set :

1. # of samples	**96**	editable. If editing # of samples also edit # of rows
2. # of plates	**1**	editable
3. # of wells for Dead vol.	**15.5**	
4. # of SourceBrewWells	**12**	
5. # of rows	**8**	editable. If editing # of rows also edit # of samples

	Sol #	ul / well	Samples Brew	DV Brew	Total volume brew	( )	Check off each
5X Second Strand buffer		**10**	960	155.2	1115.2	( )	5X Second strand buffer
Gene Amp mix with dUTP (12.5mM)		**1.5**	144	23.28	167.3	( )	Gene Amp mix with dUTP (12.5mM)
E.Coli DNA Ligase (10U/uL)		**0.5**	48	7.76	55.8	( )	E.Coli DNA Ligase (10U/uL)
E.Coli DNA Polymerase (10U/uL)		**1.5**	144	23.28	167.3	( )	E.Coli DNA Polymerase (10U/uL)
E.Coli Rnase H (2U/uL)		**0.5**	48	7.76	55.8	( )	E.Coli Rnase H (2U/uL)
DEPC water		**1**	96	15.52	111.5	( )	DEPC water
Total volume (ul)		**15**	1440	232.8	1672.8		ul Brew Mix in tube

Aliquot 133 uL of the brew above into one row, 12 wells of a plate

Appendix D : LIMS

1. Sign on to LIMS. Make sure you are under the Lib_ Construction tab. Scan in the "tra" plate barcode. Save plate set and record the number in your lab notebook. Alternatively if already exists, grab plate set number.

2. From the Protocol drop-down menu, select "B-Strand Specific cDNA Synthesis". Click "Continue Prep".

3. Under Plate RNA Strategy, select "Strand Specific". Click "Completed Step".

4. For the next 4 steps, scan in the sol numbers for

   4.1. Random Hexamers
   4.2. Strand Specific 1st strand cDNA synthesis brew
   4.3. Superscript II RT
   4.4. DEPC water

5. Perform LIMs transfer step for the XP bead clean up. Retrieve new barcode from the printer.

6. Scan in the Strand Specific 2nd Strand Synthesis brew barcode and click "Completed step".

7. Perform LIMS transfer step for the XP bead clean up. Retrieve new barcode from the printer.