FL-Ovation™ cDNA Biotin Module V2

Catalog # 4200-12, 4200-60, 4200-A01

USER GUIDE

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I. Introduction

A. Background

NuGEN's proprietary fragmentation and labeling process (patent pending), combines enzymatic and chemical processes for fragmentation of amplified single-stranded cDNA to generate labeled targets suitable for hybridization to Affymetrix GeneChip® arrays.

The FL-Ovation™ cDNA Biotin Module V2 is validated for use with amplified cDNA generated using either the 3'-initiated Ovation™ RNA Amplification System V2 (Cat.# 3100), or the WT-Ovation™ Pico RNA Amplification System (Cat.# 3300), or the WT-Ovation™ FFPE System (Cat.# 3400) . The resulting fragmented and labeled single-stranded cDNA targets generated with the FL-Ovation™ cDNA Biotin Module V2 are suitable for hybridization to Affymetrix GeneChip® arrays.

B. Fragmentation and Labeling Process

This novel and proprietary two-step fragmentation and labeling process is carried out by a simple "add and incubate" procedure and does not require purification steps.

The first step is a combined chemical and enzymatic fragmentation process that yields single-stranded cDNA products in the 50-100 base range. In the second step, this fragmented product is labeled via enzymatic attachment of a biotin-labeled nucleotide to the 3-hydroxyl end of the fragmented cDNA generated in the first step.

C. Performance Specifications

The fragmentation and biotin labeling process is performed in approximately 2 hours and produces fragmented and labeled single-stranded cDNA ranging from 50-100 bases ready for hybridization to GeneChip® arrays.

D. Quality Control

Each FL-Ovation™ cDNA Biotin Module V2 lot is tested to meet specifications for product size and array performance.

E. Storage and Stability

The FL-Ovation™ cDNA Biotin Module V2 is shipped on dry ice and should be unpacked immediately upon receipt. All components should be stored at –20 °C on internal shelves of a freezer without a defrost cycle.

Kits handled and stored according to the above guidelines should perform to specifications for 6 months. NuGEN does not recommend long-term storage of this product beyond 6 months.

F. Material Safety Data Sheets (MSDS)

An MSDS for this product is available from NuGEN Technical Service by calling 888-654-6544 or by sending an email to: techserv@nugeninc.com.

II. Kit Components

A. Reagents and Supplies Provided

Table 1. cDNA Fragmentation and Biotin Labeling Reagents

Component	4200-12 Part Number	4200-60 Part Number	4200-A01 Part Number	Vial Cap	Vial Number
Fragmentation Buffer Mix	S01168	S01182	S01177	Orange	FL1
Fragmentation Enzyme Mix	S01175	S01183	S01178	Orange	FL2
Labeling Buffer Mix	S01171	S01184	S01179	Orange	FL3
Biotin Reagent	S01172	S01185	S01180	Orange	FL4
Labeling Enzyme Mix	S01173	S01186	S01181	Orange	FL5

B. Additional Equipment, Reagents and Labware

1. Required materials

- **Equipment**
  - Microcentrifuge for individual 1.5 ml and 0.5 ml tubes
  - Microcentrifuge for 0.2 ml individual and 8 x 0.2 ml strip PCR tubes (e.g. PGC #16-7009-70/72 or similar)
  - 0.5 - 10 µl pipette , 2 - 20 µl pipette, 20 - 200 µl pipette, 200 - 1000 µl pipette
  - Vortexer
  - Thermal cycler with 0.2 ml tube heat block, heated lid, and 100 µl reaction capacity
  - Appropriate spectrophotometer and cuvettes, or Nanodrop® ND-1000 UV-Vis Spectrophotometer

- **Labware**
  - Nuclease-free pipette tips
  - 1.5 ml and 0.5 ml RNase-free microcentrifuge tubes
  - 0.2 ml individual thin wall PCR tubes or 8 x 0.2 ml strip PCR tubes
  - Appropriate spectrophotometer cuvettes
  - Disposable gloves
  - Kimwipes
  - Filter paper
  - Ice bucket

2. Optional equipment

- Agilent 2100 bioanalyzer or other equipment for electrophoretic analysis of RNA
- Real time PCR system

III. Planning the Experiment

A. Input cDNA Requirements

1. cDNA source

The most important requirement for achieving successful results with the FL-Ovation™ cDNA Biotin Module V2 is to use cDNA generated with one of NuGEN's Ovation™ Amplification System products that have been designed and validated for use with this module.

Note: The FL-Ovation™ cDNA Biotin Module V2 will not perform with cDNA prepared using any other amplification approaches other than those listed below.

The FL-Ovation™ cDNA Biotin Module V2 is validated for use with amplified cDNA generated by either the 3'-initiated Ovation™ RNA Amplification System V2 (Cat.# 3100) or the WT-Ovation™ Pico RNA Amplification System (Cat.# 3300), or the WT-Ovation™ FFPE System (Cat.# 3400). To generate the amplified cDNA follow the user guides for one of the validated NuGEN amplification kits. The unlabeled, cDNA product should be stored at –20°C, with minimum freeze thaw cycles prior to fragmentation and labeling. For recommendations on the input cDNA quality assessment, see Appendix B, and C of this user guide. You may also chose to qualify the starting cDNA by performing qPCR assays as recommended in the appropriate NuGEN Amplification System user guides.

2. cDNA purity

The cDNA input for the FL-Ovation™ cDNA Biotin Module V2 must be purified using the purification methods recommended in the user guides of the NuGEN amplification System products. The adjusted 260/280 absorbance ratio of the purified SPIA™ cDNA must be >1.8.

B. Using Nuclease-free Techniques

Nuclease contamination from equipment and work environment will lead to experimental failure. Follow these guidelines to minimize contamination:

- Wear disposable gloves and change them frequently.
- Avoid touching surfaces or materials that could introduce DNases.
- Use only the reagents provided and recommended.
- Clean and decontaminate work areas and instruments, including pipettes, with commercially available decontamination reagents.
- Use only new DNase-free pipette tips and microcentrifuge tubes.

C. Amplified Input cDNA Storage

The unlabeled, cDNA product generated by the validated NuGEN Amplification System products such as the Ovation™ RNA Amplification System V2, or WT-Ovation™ Pico System may be stored at –20°C for at least 6 months prior to fragmentation and labeling.

D. Fragmented and Labeled cDNA Storage

The fragmented and biotin-labeled cDNA product can be used immediately after preparation, or may be stored at –20 °C.

IV. Protocol

A. Overview

The cDNA fragmentation and biotin labeling is performed in two stages:

1. cDNA fragmentation	35 minutes
2. Biotin attachment	70 minutes
Total time to fragment and label amplified cDNA	**105 minutes**

B. Protocol Notes

- Thaw only components used in each step and immediately place them on ice.
- Always keep thawed reagents and reaction tubes on ice unless otherwise instructed.
- After thawing and mixing buffer mixes, if any precipitate is observed, re-dissolve it completely prior to use. You may gently warm the buffer mix for 2 minutes at room temperature followed by brief vortexing. Do not warm any enzyme mixes.
- The reagent volumes recovered greatly depend on the number of batched process with each kit. Set up no fewer than three reactions at a time with the 4200-12 kit, no fewer than 10 reactions at a time with 4200-60, and no fewer than 48 reactions at a time with 4200-A01. The A01 kit has been designed for use with an automation protocol requiring large batch sizes, for more information about NuGEN's automation solutions contact our technical support team.
- When placing small amounts of reagents into reaction mix, gently pipette up and down several times to ensure complete transfer.
- When instructed to pipette mix, gently aspirate and dispense a volume, at least half of total reaction mix volume. Repeat a minimum of five times to ensure complete mixing.
- Allow thermal cycler to reach incubation temperature before placing samples in the block.
- When working with more than one sample, excess master mix may be needed.
- Components of this NuGEN product should not be used or combined with any other types of Ovation™ System products and vice versa.

C. Preparing cDNA Samples

The amount of amplified cDNA required for each fragmentation and labeling reaction depends on the method of cDNA generation. NuGEN's various amplification Systems validated for use with the FL-Ovation™ cDNA Biotin Module V2 and the required cDNA input from each are listed in Table 2. Ensure that the correct input is used in section IV.E., step 6 of the Fragmentation Protocol.

Table 2. cDNA input requirements for fragmentation and labeling reactions

NuGEN Amplification System (Cat. #)	cDNA input per reaction
WT-Ovation™ FFPE System (Cat.# 3400)	5 µg
WT-Ovation™ Pico RNA Amplification System (Cat.# 3300)	5 µg
Ovation™ RNA Amplification System V2 (Cat.# 3100)	3.75 µg
Whole Blood Solution and the Ovation™ WB Reagent (Cat.# 1300)	4.4 µg

D. Programming the Thermal Cycler

Use a thermal cycler with a heat block designed for 0.2 ml tubes, equipped with a heated lid, and with a capacity of 100 µl reaction volume. Prepare the 2 programs shown in Table 3, following the operating instructions provided by the manufacturer. For thermal cyclers with an adjustable heated lid, set the lid temperature at 100 °C. For thermal cyclers with a fixed temperature heated lid (e.g. ABI GeneAmp® PCR 9600 and 9700 models) use the default settings (typically 100 to 105 °C).

Table 3. Thermal Cycler Programming

PROGRAMMING DETAILS	
Program 1: cDNA Fragmentation	37 °C for 30 minutes, 95 °C for 2 minutes, then 4 °C forever
Program 2: Labeling	37 °C for 60 minutes, 70 °C for 10 minutes, then 4 °C forever

E. Fragmentation Protocol

1. Obtain the Fragmentation Buffer Mix (Orange: FL1) and Fragmentation Enzyme Mix (Orange: FL2) from the product box stored at -20 °C. **Note**: you may thaw all reagents at once, see Labeling protocol section for thawing and mixing instructions for the Labeling reagents.
2. Thaw FL1 at room temperature and mix by vortexing for two seconds and then spin in a microcentrifuge for two seconds. Then, place on ice.
3. Mix FL2 by inverting the tube three times. Spin tube in microcentrifuge for 2 seconds. Then, place on ice.
4. Make Fragmentation Master Mix as outlined below:

Use Fragmentation Master Mix immediately after preparation.

Table 4. Fragmentation Master Mix (volumes listed are for a single reaction)

FRAGMENTATION BUFFER MIX (ORANGE: FL1)	FRAGMENTATION ENZYME MIX (ORANGE: FL2)
5 µl	2 µl

Note: Mix by pipetting and spin down the master mix briefly. Place on ice. Use master mix immediately.

5. Place 0.2 ml PCR tube(s) in a rack on ice.
6. For each reaction, pipet 25 µl ***(refer to Table 2 to determine the amount of required amplified cDNA input)*** of the purified SPIA™ cDNA into a PCR tube. Add water, if necessary, to bring up the volume of samples to 25 µl.
7. Add 7 µl of the Fragmentation Master Mix to each sample.
8. Mix well by pipetting up and down 8-10 times.
9. Cap tubes; vortex and spin for 2 seconds to ensure thorough mixing.
10. Place tubes in a pre-warmed thermal cycler programmed to run Program 1 (cDNA Fragmentation, see Table 3):
    a. Incubate at 37 °C for 30 minutes.
    b. Incubate at 95 °C for 2 minutes.
    c. Cool to 4 °C

11. Remove tubes from the thermal cycler and spin for 2 seconds to collect condensation, then place on ice. Proceed immediately to the Labeling step.

F. Labeling Protocol

1. Obtain the Labeling Buffer Mix (Orange: FL3), and Labeling Reagent (Orange: FL4), and the Labeling Enzyme Mix (Orange: FL5) from the product box stored at -20 °C.
2. Place all reagents immediately on ice.
3. Thaw FL3 and FL4 at room temperature and mix by vortexing for two seconds and then spin in a microcentrifuge for two seconds. Then, place on ice.
4. Mix FL5 by inverting the tube three times. Spin tube in microcentrifuge for 2 seconds. Then, place on ice.
5. Make Labeling Master Mix as outlined below:

Use Labeling Master Mix immediately after preparation.

Table 5. Labeling Master Mix (volumes listed are for a single reaction)

Labeling Buffer Mix (Orange: FL3)	Labeling Reagent (Orange: FL4)	Labeling Enzyme Mix (Orange: FL5)
15 µl	1.5 µl	1.5 µl

Note: Mix by pipetting and spin down the master mix briefly. Place on ice. Use master mix immediately.

6. Place 0.2 ml PCR tube(s) in a rack on ice.
7. Add 18 µl of the Labeling Master Mix to each fragmented cDNA sample tube.
8. Mix well by pipetting up and down 8-10 times.
9. Cap tubes; vortex and spin for 2 seconds to ensure thorough mixing.
10. Place tubes in a pre-warmed thermal cycler programmed to run Program 2 (Labeling, see Table 3):
    a. Incubate at 37 °C for 60 minutes.
    b. Incubate at 70 °C for 10 minutes.
    c. Cool to 4 °C.
11. After completion, remove tubes from thermal cycler and spin for 2 seconds to collect condensation.
12. The fragmented and labeled cDNA may be processed immediately for array hybridization or stored at -20 °C. For recommendations on array hybridization, see Appendix A.

V. Technical Support

For Technical Support, please contact NuGEN at (US only) 888.654.6544 (Toll-Free Phone) or 888.296.6544 (Toll-Free Fax) or email techserv@nugeninc.com.
In Europe contact NuGEN at +31(0)135780215 (Phone) or +31(0)135780216(Fax) or email europe@nugeninc.com.
In all other locations, contact your NuGEN distributor's Technical Support team.

VI. Appendix

A. Target Preparation for Affymetrix GeneChip® Analysis

Targets containing amplified, fragmented and biotin-labeled cDNA generated using the FL-Ovation™ cDNA Biotin Module V2 are prepared for analysis on GeneChip® standard arrays according to the Affymetrix GeneChip® Expression Analysis Technical Manual (revision 4). Components and supply sources used in the hybridization cocktail are as specified in the manual, however there are a few exceptions and minor differences in the protocol that are outlined below.

To prepare target for a single standard array, use a 1.5 ml microcentrifuge tube and mix at room temperature the amount of target cDNA and volumes of hybridization cocktail components indicated in the Table 6 below. Heat denature the hybridization cocktail at 99°C for 2 minutes (not 5 minutes as specified by Affymetrix) then follow the Affymetrix standard protocol (45°C in a heat block for 5 minutes then centrifuge at maximum speed for 5 minutes just prior to loading). For a standard GeneChip® array, use a 200-µl volume and for a Midi array use 130 µl. NuGEN recommends hybridization time of 18 hours. Hybridization for 16 to 20 hours yields comparable results. Use protocol EukGE-WS2v4_450 for standard arrays or the Midi_Euk-2v3 for Midi arrays on the GeneChip® Fluidics Station 450.

Table 6. Hybridization Cocktail Assembly for Single Standard GeneChip® Array

COMPONENT	STANDARD ARRAY (49 FORMAT)	MIDI ARRAY (100 FORMAT)	FINAL CONCENTRATION
Fragmented, biotin-labeled amplified cDNA	50 µl	34 µl	Depends on how the cDNA is generated*
Control oligonucleotide B2 (3 nM)	3.7 µl	2.5 µl	50 pM
20X Eukaryotic hybridization controls (bioB, bioC, bioD, cre)	11 µl	7.5 µl	1.5, 5, 25 and 100 pM respectively
Herring sperm DNA (10 mg/ml)	2.2 µl	1.5 µl	0.1 mg/ml
Acetylated BSA (50 mg/ml)	2.2 µl	1.5 µl	0.5 mg/ml
2x Hybridization buffer	110 µl	75 µl	1x
100% DMSO	22 µl	15 µl	10%
H_2O	19 µl	13 µl	N/A
Final Volume	**220 µl**	**150 µl**	

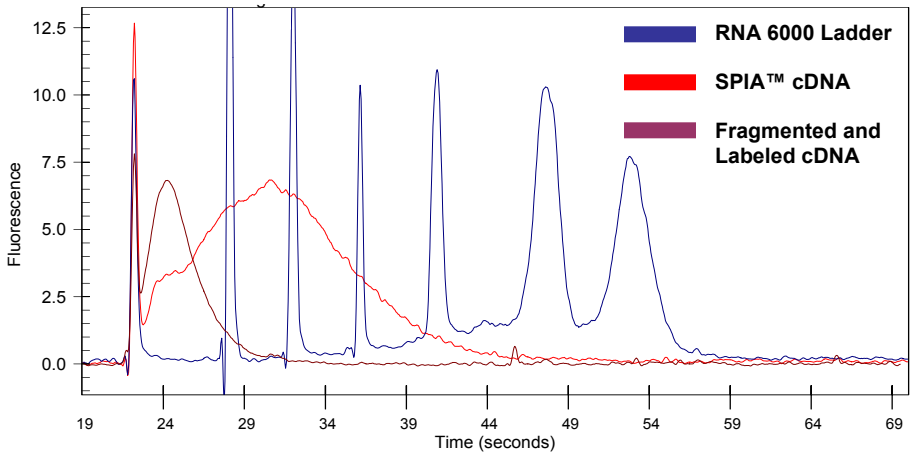
* Refer to Table 2 for cDNA input requirements into fragmentation and labeling reactions.

B. Quality Control of Amplified, Fragmented and Labeled cDNA Product

The fragmentation success and the size distribution of the final fragmented and biotinylated product may be viewed on an Agilent Bioanalyzer, by loading 100 ng of each sample before and after the fragmentation and labeling process on an RNA 6000 Nano LabChip® (Agilent Cat.# 5065-4476) using the mRNA Smear Nano program following the manufacturer's instructions. Product that is not sufficiently fragmented has been shown to yield poor results on GeneChips®

arrays. For good results on GeneChip® arrays, 80% or greater of the fragmented cDNA product should be smaller than 200 bases in length. For examples of Bioanalyzer traces of unfragmented and fragmented cDNA product, see Figure below.

Figure 1. Bioanalyzer Trace of Amplified, un-fragmented and fragmented cDNA Product: HeLa RNA amplified with the WT-Ovation™ Pico System (Cat.# 3300) was processed with the FL-Ovation™ cDNA Biotin Module V2, and analyzed on an Agilent Bioanalyzer.



C. Input cDNA Analysis: Measuring Concentration and Purity

1. Before using the FL-Ovation™ cDNA Biotin Module V2, it is highly recommended to determine the concentration of your sample to ensure sufficient cDNA input for the fragmentation and labeling process.
2. Mix your sample by brief vortexing and spinning prior to checking the concentration.
3. Measure the absorbance at 260, 280 and 320 nm of your amplified cDNA product. You may need to make a 1:20 dilution of the cDNA in water prior to measuring the absorbance.
4. Purity: Subtract the Abs_{320} value from both Abs_{260} and Abs_{280} values. The adjusted ($Abs_{260} – Abs_{320}$ / $Abs_{280} – Abs_{320}$) ratio should be > 1.8.
5. Yield: Assume 1 absorbance unit at 260 nm of single-stranded DNA = 33 µg/ ml.
   To calculate:
   ($Abs_{260} – Abs_{320}$ of diluted sample) x (dilution factor) x 33 (concentration in µg/ml of a 1 absorbance unit solution) x 0.03 (final volume in ml) = total yield in micrograms
6. Alternatively you may measure the concentration and purity of cDNA with a Nanodrop, using 1 absorbance unit at 260 nm of single-stranded DNA = 33 µg/ ml as the constant.

D. Frequently Asked Questions (FAQs)

Q1. What materials are provided with the FL-Ovation™ cDNA Biotin Module V2?
The Module provides all necessary buffers and enzymes for fragmentation and labeling of cDNA generated with a validated NuGEN Amplification System.

Q2. What equipment is required or will be useful?
Required equipment includes a microcentrifuge, pipettes, vortexer, a thermal cycler, and a U.V./Vis spectrophotometer. An Agilent Bioanalyzer or a similar instrument may be used for quality control.

Q3. What additional reagents are required for the FL-Ovation™ cDNA Biotin Module V2?
No additional reagents are required.

Q4. What type of cDNA should I use with the FL-Ovation™ cDNA Biotin Module V2?
You must use SPIA™ cDNA generated with either the WT-Ovation™ Pico RNA Amplification System (Cat.# 3300) or the Ovation™ RNA Amplification System V2 (Cat.# 3100), or WT-Ovation™ FFPE System (Cat.# 3400).

Q5. How much labeled cDNA should I hybridize to a GeneChip® array?
We recommend using the entire 50 µl of the F&L reaction for a standard GeneChip® array and 34 µl for a Midi format array hybridization, see Appendix A.

Q6. Can I vary the amount of cDNA input to fragmentation and labeling?
We recommend 5 µg input for cDNA generated with either WT-Ovation™ FFPE System or the WT-Ovation™ Pico System. 3.75 µg of cDNA generated with the Ovation™ RNA Amplification System V2 (Cat.# 3100) should be used per F&L reaction. It is very important that the amount of cDNA input is kept consistent across all samples for each experiment.

Q7. Can I use any cDNA as starting material in the FL-Ovation™ cDNA Biotin Module V2?
No, the cDNA must be generated using a validated NuGEN Amplification System. Use of other cDNAs will result in poor performance.

Q8. How much fragmented and labeled cDNA yield can I expect?
Since this module does not require any purification, the total yield is equal to the input cDNA.

Q9. What is the size range of fragmented and labeled cDNA generated by the FL-Ovation™ cDNA Biotin Module V2?
As measured with an Agilent Bioanalyzer, 80% of product falls below 200 bases with an average peak at 85 bases.

Q10. Has NuGEN performed reproducibility studies on the FL-Ovation™ cDNA Biotin Module V2?
Yes, our studies have included sample to sample, lot-to-lot, and operator-to-operator reproducibility, see FL-Ovation™ cDNA Biotin Module V2 Technical Report #1 for some of these studies.

Q11. Can the FL-Ovation™ cDNA Biotin Module V2 be used for fragmentation and labeling of RNA?
No.

Q12. Should I purify the cDNA before hybridization?
No. Purification of the fragmented and labeled product is not necessary.

Q13. What are the recommended storage conditions for the fragmented and labeled cDNA?
The fragmented and labeled cDNA may be stored at -20°C. Ensure the vials are well sealed and avoid multiple freeze thaw cycles.

Q14. What types of arrays work with the FL-Ovation™ cDNA Biotin Module V2 cDNA?
The FL-Ovation™ cDNA Biotin Module V2 has been validated on Affymetrix 3' Expression GeneChip® arrays.

Q15. Are the array hybridization reagents included in the FL-Ovation™ cDNA Biotin Module V2?
No. We only provide the reagents necessary for fragmentation and labeling of cDNA. We do provide a recommended procedure for hybridization in the users guide, see Appendix A.

Q16. What hybridization and wash protocols do you recommend for Affymetrix GeneChip® applications?
We recommend the same methods as the Affymetrix protocol with the following adjustments:

a. Heat denature the hybridization cocktail at 99°C for 2 minutes
b. Hybridize chips for 16-20 hours.
c. Use the Affymetrix fluidics wash script EukGE-WS2v4_450 or the Midi_Euk-2v3or the for GeneChip® Fluidics Station 450. Do not use the version 5 wash scripts.

Q17. What are the FL-Ovation™ cDNA Biotin Module V2 incubation temperatures for each step?
cDNA Fragmentation: 37 °C for 30 minutes, then 95 °C for 2 minutes, then cool to 4 °C
cDNA Labeling: 37 °C for 60 minutes, then 70 °C for 10 minutes, then cool to 4 °C.

Q18. Where can I safely stop in the fragmentation and labeling protocol?
We do not recommend stopping at any step of the protocol.

Q19. How do I determine fragmentation success?
If you chose to determine the success of fragmentation, you may use the Agilent Bioanalyzer to inspect the size distribution of samples before and after fragmentation, see Appendix B

Q20. How should I qualify my cDNA for use with the FL-Ovation™ cDNA Biotin Module V2?
You must use cDNA generated with a validated NuGEN Amplification System product. The concentration of starting cDNA must be determined to ensure adequate input into the F&L reaction and therefore onto the arrays, see table 2 in this user guide for cDNA input requirements. You may chose to further qualify the starting cDNA by performing qPCR assays as recommended in the appropriate NuGEN Amplification System user guides.

Notes

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