

WHOLE / TRANSCRIPTOME / WT-OVATION™

WT-Ovation™ Exon Module VERSION 1.0 Catalog # 2000-12, 2000-60

USER GUIDE



NuGEN™

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Table of Contents

<u>I.</u>	<u>Introduction</u>	1
A.	Background	1
B.	ST-cDNA Generation Process	1
C.	Performance Specifications	1
D.	Quality Control	1
E.	Storage and Stability	1
F.	Material Safety Data Sheets (MSDS)	1
<u>II.</u>	<u>Kit Components</u>	2
A.	Reagents and Supplies Provided	2
B.	Additional Equipment, Reagents and Labware	2
<u>III.</u>	<u>Planning the Experiment</u>	3
A.	Input cDNA Requirements	3
B.	Using Nuclease-free Techniques	3
C.	Amplified Input cDNA Storage	3
D.	ST-cDNA Storage	3
<u>IV.</u>	<u>Protocol</u>	4
A.	Overview	4
B.	Protocol Notes	4
C.	Programming the Thermal Cycler	4
D.	ST-cDNA Generation Protocol	5
E.	ST-cDNA Purification Protocol	5
F.	Measuring ST-cDNA Product Yield and Purity	6
<u>V.</u>	<u>Technical Support</u>	7
<u>VI.</u>	<u>Appendix</u>	8
A.	Input cDNA Analysis: Measuring Concentration and Purity	8
B.	Alternate ST-cDNA cleanup instructions	8
C.	Fragmentation and labeling using the FL-Ovation™ cDNA Biotin Module	9
D.	Frequently Asked Questions (FAQs)	10

I. Introduction

A. Background

NuGEN's sense transcript cDNA (ST-cDNA) generation process utilizes a robust enzymatic methodology to generate ST-cDNA suitable for fragmentation and labeling as targets for Affymetrix GeneChip® Exon and Gene ST arrays.

The WT-Ovation™ Exon Module is validated for use with amplified cDNA generated using either the WT-Ovation Pico RNA Amplification System (Cat.# 3300) or the WT-Ovation FFPE System (Cat.# 3400).

B. ST-cDNA Generation Process

The ST-cDNA generation process is carried out by a simple “add and incubate” procedure and requires a single final purification step. The first step allows the primer to anneal to the single-stranded cDNA target. The second step utilizes DNA polymerase to extend the annealed primer and generate double-stranded cDNA.

C. Performance Specifications

The process is performed in approximately 2 hours and produces ST-cDNA ready for fragmentation and labeling with the FL-Ovation™ cDNA Biotin Module V2. 3 µg of SPIA™ cDNA should produce more than 5 µg of ST-cDNA, a quantity sufficient for one Exon ST array hybridization.

D. Quality Control

Each WT-Ovation™ Exon Module lot is tested to meet product specifications.

E. Storage and Stability

The WT-Ovation™ Exon Module 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. ST-cDNA Reagents

COMPONENT	2000-12 PART NUMBER	2000-60 PART NUMBER	VIAL CAP	VIAL NUMBER
Primer Mix	S01238	S01241	Yellow	E1
Buffer Mix	S01239	S01242	Yellow	E2
Enzyme Mix	S01240	S01243	Yellow	E3
Nuclease-free Water	S01001	S01113	Green	D1

B. Additional Equipment, Reagents and Labware

1. Required materials

o Equipment

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

o Labware

- o Nuclease-free pipette tips
- o 1.5 ml and 0.5 ml RNase-free microcentrifuge tubes
- o 0.2 ml individual thin wall PCR tubes or 8 x 0.2 ml strip PCR tubes
- o DNA Clean & Concentrator™-25 (Zymo Research, Cat. # D4005)
- o Disposable gloves
- o Kimwipes
- o Ice bucket

2. Optional equipment

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

To Order:

- o Zymo Research, (888) 882-9682, www.zymoresearch.com

III. Planning the Experiment

A. Input cDNA Requirements

1. cDNA source

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

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

The WT-Ovation™ Exon Module is validated for use with amplified cDNA generated by either 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 may be stored at –20°C, with minimum freeze thaw cycles prior to ST-cDNA generation. For recommendations on the input cDNA quality assessment, see Appendix A and B of this user guide. You may also qualify the starting cDNA by performing qPCR assays recommended in the appropriate Amplification System user guides.

2. cDNA purity

The cDNA used as input for the WT-Ovation™ Exon Module must be purified using one of the 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.

3. cDNA input amount

The amount of cDNA required as input into the Exon Module is 3 µg. For FFPE or degraded samples, we recommend using 4 µg of input cDNA.

B. Using Nuclease-free Techniques

Nuclease contamination from equipment and the 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, generated by a validated NuGEN Amplification System product such as the WT-Ovation™ FFPE System or the WT-Ovation™ Pico System, may be stored at –20°C for at least 6 months prior to ST-cDNA generation.

D. ST-cDNA Storage

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

IV. Protocol

A. Overview

The ST-cDNA generation process is performed in three stages:

- | | |
|-----------------------------|------------|
| 1. Primer annealing | 5 minutes |
| 2. ST-cDNA generation | 80 minutes |
| 3. Cleanup and quantitation | 15 minutes |

Total time to purified ST-cDNA	100 minutes
---------------------------------------	--------------------

B. Protocol Notes

- 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 batches processed with each kit. Set up no fewer than three reactions at a time with the 2000-12 kit, no fewer than 10 reactions at a time with 2000-60.
- When placing small amounts of reagent 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 of at least half of the total reaction mix volume. Repeat a minimum of five times to ensure complete mixing.
- Allow thermal cycler to reach the initial 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. 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 100 µl reaction volume capacity. Prepare the 2 programs shown in Table 2 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 2. Thermal Cycler Programming

PROGRAMMING DETAILS	
Program 1: Primer Anneal	95 °C for 5 minutes, then 4 °C forever
Program 2: ST-cDNA Generation	4 °C for 1 minute, 30 °C for 10 minutes, 42 °C for 60 minutes, 75 °C for 10 minutes, then 4 °C forever

D. ST-cDNA Generation Protocol

1. Obtain the Primer Mix (Yellow: E1), Buffer Mix (Yellow: E2) and Enzyme Mix (Yellow: E3) from the product box stored at -20 °C.
2. Thaw E1 and E2 at room temperature and mix by vortexing for two seconds and then spin in a microcentrifuge for two seconds. Place on ice.
3. Mix E3 by inverting the tube three times, spin in microcentrifuge for 2 seconds, then place on ice.
4. Place 0.2 ml PCR tube(s) in a rack on ice.
5. For each reaction, pipette 3 µg purified cDNA (or 4 µg of cDNA from FFPE/degraded samples) generated by a validated NuGEN amplification system (see Section III.A.1. above) into a 0.2 ml PCR tube. Add nuclease-free water, if necessary, to bring up the final volume to 20 µl.
6. To each reaction tube add 6 µl E1 Primer Mix and mix well by pipetting up and down 8-10 times.
7. Close the cap on each reaction tube tightly, spin down briefly and place the tubes in a thermal cycler programmed to run Program 1 (Primer Anneal, see Table 2)
 - a. 95 °C for 5 minutes
 - b. Cool to 4 °C
8. Remove PCR tubes from thermal cycler and spin down briefly in a microcentrifuge.
9. Make ST-cDNA Generation Master Mix as outlined below:

Table 3. ST-cDNA Master Mix (volumes listed are for a single reaction)

BUFFER MIX (YELLOW: E2)	ENZYME MIX (YELLOW: E3)
38 µl	6 µl

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



Use Master Mix immediately after preparation.

10. Add 44 µl of the ST-cDNA Master Mix to each sample.
11. Mix well by pipetting up and down 8-10 times.
12. Cap tubes, vortex and spin for 2 seconds to ensure thorough mixing.
13. Place tubes in a pre-warmed thermal cycler programmed to run Program 2 (ST-cDNA Generation, see Table 3):
 - a. Incubate at 4 °C for 1 minute
 - b. Incubate at 30 °C for 10 minutes
 - c. Incubate at 42 °C for 60 minutes
 - d. Incubate at 75 °C for 10 minutes
 - e. Cool to 4 °C
14. Remove tubes from the thermal cycler, spin for 2 seconds to collect condensation, then place on ice. ST-cDNA may be stored at -20 °C or carried on to the purification step.

E. ST-cDNA Purification Protocol

1. Obtain the appropriate number of Zymo II columns from the DNA Clean & Concentrator™-25 kit.
2. Place 200 µl of DNA Binding Buffer in a clean 1.5 ml tube.
3. Add the entire ST-cDNA reaction volume (70 µl) to the tube.
4. Vortex and spin down briefly.

5. For each sample, place one Zymo-Spin II column into a collection tube.
6. Load the entire volume of sample (270 µl) onto the Zymo-Spin II column.
7. Centrifuge column in the collection tube for 10 seconds at >10,000 x g in a microcentrifuge.
8. Discard flow-through. Place the Zymo-Spin II column back in the same collection tube.
9. Wash sample by adding 200 µl of room temperature 80% ethanol. Do not use the Wash Buffer provided with the Zymo columns. **Note:** Use fresh 80% ethanol. Lower percent ethanol mixes will reduce recovery.
10. Centrifuge column in the collection tube for 10 seconds at >10,000 x g in a microcentrifuge. Discard flow-through.
11. Add 200 µl of room temperature 80% ethanol.
12. Centrifuge column in the collection tube for 30 seconds at >10,000 x g in a microcentrifuge. Discard flow-through.
13. Place the Zymo-Spin II Column in a clean 1.5 ml microcentrifuge tube.
14. Add 30 µl of room temperature nuclease-free water (green: D1) from the kit to the center of each Zymo-Spin II column. Do not use cold water!
15. Let columns stand for 1 minute at room temperature.
16. Centrifuge column and microcentrifuge tube for 30 seconds at >10,000 x g in a microcentrifuge.
17. Collect sample. There should be approximately 30 µl of purified cDNA.
18. Mix sample by vortexing, then spin briefly.
19. Proceed to Measuring ST-cDNA Product Yield and Purity step.



Use nuclease-free water at room temperature to elute sample.

F. Measuring ST-cDNA Product Yield and Purity

1. Mix your sample by brief vortexing and spinning prior to checking the concentration.
2. Measure the absorbance of your amplified cDNA product at 260, 280 and 320 nm. You may need to make a 1:20 dilution of the cDNA in water prior to measuring the absorbance.
3. Purity: Subtract the Abs₃₂₀ value from both Abs₂₆₀ and Abs₂₈₀ values. The adjusted $(Abs_{260} - Abs_{320} / Abs_{280} - Abs_{320})$ ratio should be > 1.8.
4. Yield: Assume 1 absorbance unit at 260 nm of ST-cDNA = **33** µg/ ml.
To calculate:
 $(Abs_{260} - Abs_{320} \text{ of diluted sample}) \times (\text{dilution factor}) \times 33 \text{ (concentration in } \mu\text{g/ml of a 1 absorbance unit solution)} \times 0.03 \text{ (final volume in ml)} = \text{total yield in micrograms}$
5. Alternatively you may measure the concentration and purity of ST-cDNA with a Nanodrop, using 1 absorbance unit at 260 nm of ST-cDNA = **33** µg/ ml as the constant.
6. The purified ST-cDNA may be stored at -20°C or fragmented and labeled with the FL-Ovation™ cDNA Biotin Module V2(Cat. #4200).

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. Input cDNA Analysis: Measuring Concentration and Purity

1. Before using the WT-Ovation™ Exon Module, it is highly recommended to determine the concentration of your sample to ensure sufficient cDNA input for the ST-cDNA generation process.
2. Mix your sample by brief vortexing and spinning prior to checking the concentration.
3. Measure the absorbance of your amplified cDNA product at 260, 280 and 320 nm. You may need to make a 1:20 dilution of the cDNA in water prior to measuring the absorbance.
4. Purity: Subtract the Abs₃₂₀ value from both Abs₂₆₀ and Abs₂₈₀ 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.
 - a. To calculate:
 - b. $(Abs_{260} - Abs_{320} \text{ of diluted sample}) \times (\text{dilution factor}) \times 33 \text{ (concentration in } \mu\text{g/ml of a 1 absorbance unit solution)} \times 0.03 \text{ (final volume in ml)} = \text{total yield in micrograms}$
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.

B. Alternate ST-cDNA cleanup instructions

Agencourt RNAClean® magnetic beads (**instructions for a single reaction**)

1. Obtain and vigorously shake the RNAClean® bottle to resuspend the magnetic beads.
2. Place all of the 70 µl ST-cDNA into a clean 0.2 ml tube or PCR plate well.
3. Add 126 µl of resuspended RNAClean® beads to the 70 µl ST-cDNA sample.
4. Mix the sample and beads thoroughly by pipetting up and down 10 times.
5. Incubate samples at room temperature for 5 minutes.
6. Place the samples on the plate magnet for 10 minutes or until solution clears.
7. Remove and discard the supernatant. Do not disturb the ring of magnetic beads.
8. With the samples still on the plate magnet, add 200 µl of freshly prepared 80% ethanol to each sample and incubate for 30 seconds or until the solution clears. Add slowly as to not disturb the separated magnetic beads.
9. Remove and discard the ethanol.
10. Repeat the 80% ethanol wash 2 more times. Ensure all the ethanol is removed.
11. Air dry the samples on bench top off the plate magnet for 10-20 minutes, ensuring samples are completely dry before proceeding. Remove any remaining ethanol using a pipette.
12. With the samples on bench top, add 30 µl of the RNase-free Water to each sample. Vortex samples for 30 seconds or use a plate shaker set to medium speed. Ensure the beads are fully resuspended, vortex longer if necessary.
13. Replace samples back on the plate magnet; allow the beads to separate for 5 minutes or until the solution clears.
14. Remove the eluted sample and place into a fresh tube, avoiding any bead carry-over. There should be approximately 30 µl of purified cDNA.

Note: Small amounts of magnetic bead carry-over may interfere with sample quantitation take care to minimize bead carry-over.
15. Mix sample by vortexing, spin briefly, and proceed to measuring cDNA yield.



Use nuclease-free water at room temperature to elute sample.

C. Fragmentation and labeling using the FL-Ovation™ cDNA Biotin Module

1. Purified ST-cDNA should be fragmented and labeled using FL-Ovation™ cDNA Biotin Module V2 (Cat. #4200). For hybridization, follow the instructions in the FL-Ovation™ cDNA Biotin Module V2 User guide or the description below.
2. Use 5 µg of ST-cDNA and follow the FL-Ovation™ cDNA Biotin Module V2 User Guide recommendations to obtain fragmented and labeled cDNA. Targets are prepared for analysis on GeneChip® arrays according to the Affymetrix GeneChip® Expression Analysis Technical Manual (P/N 702232 Rev. 2).

To prepare target for a single 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 Table 4 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). In the meantime, incubate the probe array filled with Pre-Hybridization Mix at 45°C for 10 minutes with rotation. For the 49 format and 169 format GeneChip® arrays, use 200 µl and 90 µl hybridization volumes, respectively. NuGEN recommends hybridization time of 18 hours. Hybridization for 16 to 20 hours yields comparable results. For Affymetrix GeneChip ST Exon Arrays and ST Gene Arrays, use fluidics protocols FS450_0001 and FS450_0007, respectively (See Table 4).

Table 4. Hybridization, Cocktail Assembly and Fluidics Protocols for Single GeneChip® ST Arrays using Affymetrix Hybridization Wash Stain (HWS) kit (Affymetrix P/N 900720)

COMPONENT	STANDARD EXON ARRAY (49 FORMAT)	MINI GENE ARRAY (169 FORMAT)	FINAL CONCENTRATION
Fragmented, biotin-labeled amplified cDNA	50 µl	25 µl	22.73 ng/µl
Control oligonucleotide B2 (3 nM)	3.7 µl	1.8 µl	50 pM
20X Eukaryotic hybridization controls (bioB, bioC, bioD, cre)	11 µl	5.5 µl	1.5, 5, 25 and 100 pM respectively
2x Hybridization buffer	110 µl	55 µl	1x
100% DMSO	22 µl	11 µl	10%
Water	23.3 µl	11.6 µl	N/A
Final Volume	220 µl	110 µl	
Fluidics protocols	FS450_0001	FS450_0007	

D. Frequently Asked Questions (FAQs)

Q1. What materials are provided with the WT-Ovation™ Exon Module?

The Module provides all necessary buffers and enzymes for converting cDNA generated with a validated NuGEN Amplification System into sense target cDNA (ST-cDNA).

Q2. What equipment is required or will be useful?

Required equipment include a microcentrifuge, pipettes, vortexer, 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 WT-Ovation™ Exon Module?

Zymo Clean & Concentrator™-25 purification columns.

Q4. What type of cDNA should I use with the WT-Ovation™ Exon Module?

You must use SPIA™ cDNA generated with either the WT-Ovation™ Pico RNA Amplification System (Cat.# 3300) or the WT-Ovation™ FFPE System (Cat.# 3400).

Q6. Can I vary the amount of cDNA input to ST-cDNA Generation?

For RNA samples of good quality, we recommend 3 µg input for cDNA generated with either WT-Ovation™ FFPE System or the WT-Ovation™ Pico System. For RNA from FFPE or degraded RNA sources, we recommend 4 µg cDNA input. It is 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 WT-Ovation™ Exon Module?

No, the cDNA must be generated using a validated NuGEN Amplification System. Use of other cDNAs will result in poor performance.

Q8. How much ST-cDNA yield can I expect?

6-8 µg

Q9. What is the size range of ST-cDNA generated by the WT-Ovation™ Exon Module?

As measured with an Agilent Bioanalyzer, the ST-cDNA is shorter than the template cDNA.

Q10. Has NuGEN performed reproducibility studies on the WT-Ovation™ Exon Module?

Yes, our studies have included sample to sample, lot-to-lot, and operator-to-operator reproducibility. See WT-Ovation™ Exon Module Technical Report #1 for some of these studies.

Q12. Should I purify the input cDNA before ST-cDNA generation?

Yes. The protocol requires a specific quantity of amplified cDNA and the cDNA must be purified in order to be accurately quantitated.

Q13. What are the recommended storage conditions for the ST-cDNA?

The ST-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 WT-Ovation™ Exon Module cDNA?

The WT-Ovation™ Exon Module has been validated to generate cDNA targets ready for fragmentation and labeling with the FL-Ovation cDNA Biotin Module V2 prior to hybridization on Affymetrix ST arrays.

Q15. Are the fragmentation and labeling reagents included in the WT-Ovation™ Exon Module?

No. This kit only includes the reagents necessary for generating ST-cDNA. The fragmentation and labeling components are included in the FL-Ovation™ cDNA Biotin Module V2.

Q15. Are the array hybridization reagents included in the WT-Ovation™ Exon Module?

No. This kit only includes the reagents necessary for generating ST-cDNA.

Q16. What are the WT-Ovation™ Exon Module incubation temperatures for each step?

Primer Anneal: 95 °C for 5 minutes, then cool to 4 °C

ST-cDNA Generation: 4 °C for 1 minute, 30 °C for 10 minutes, 42 °C for 60 minutes, 70 °C for 10 minutes, then 4 °C forever.

Q17. Where can I safely stop in the ST-cDNA generation protocol?

You may stop after the second incubation step (ST-cDNA generation) just before ST-cDNA purification.

Q20. How should I qualify my cDNA for use with the WT-Ovation™ Exon Module?

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 ST-cDNA reaction. You may choose to further qualify the starting cDNA by performing qPCR assays as recommended in the appropriate NuGEN Amplification System user guides.

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