Targeted ExSeq -- Sequencing Library Preparation

Nov 29, 2021

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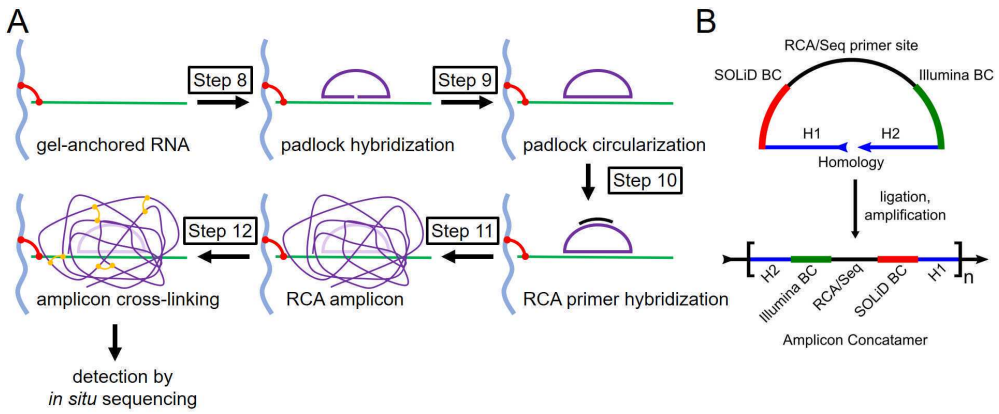

Figure 1. **(A)** Summary of steps described in this protocol. **(B)** Sequence-level description of library preparation.

This protocol accompanies Expansion Sequencing (ExSeq), describing the process of targeted ExSeq library preparation for a sample that has been processed according to a Targeted ExSeq Tissue Preparation protocol. The steps described here are a generalization of the protocol used in figures 4-6 of the paper, and represent our recommendations for future users of the technology.

The flowchart in **Fig. 1A** depicts the library preparation workflow. **Fig. 1B** is a summary of the product, in which padlock probes are amplified to form amplicon concatamers. The net result of the process is that barcode sequences are delivered to transcripts of interest and locally amplified hundreds to thousands of times.

The process of library preparation encompasses the following steps. (1) Oligonucleotide padlock probes bearing barcode sequences hybridize to RNA transcripts of interest (Step 8). (2) SplintR Ligase, which can has RNA-splinted DNA ligase activity, ligates adjacent ends of padlock probes, forming circular DNA molecules (Step 9). (3) A universal primer hybridizes to all padlock probes (Step 10). (4) Rolling Circle Amplification initiates from the primers, and repeatedly copies the sequence of the padlock probes, forming an amplicon (Step 11). (5) BS(PEG)9 covalently cross-links the amplicon to itself, stabilizing the amplicon (Steps 12-13) during *in situ* sequencing. At this point, the sample is now ready for downstream detection (i.e. via hybridizing fluorophore-labeled oligos to the amplicon (Step 14), and *in situ* sequencing).

This protocol was used (with no modifications) to profile human metastatic breast cancer biopsies as a part of the Human Tumor Atlas Pilot Project (HTAPP).

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protocol

Alon S*, Goodwin DR*, Sinha A*, Wassie AT*, Chen F*, Daugharthy ER**, Bando Y, Kajita A, Xue AG, Marrett K, Prior R, Cui Y, Payne AC, Yao CC, Suk HJ, Wang R, Yu CJ, Tillberg P, Reginato P, Pak N, Liu S, Punthambaker S, Iyer EPR, Kohman RE, Miller JA, Lein ES, Lako A, Cullen N, Rodig S, Helvie K, Abravanel DL, Wagle N, Johnson BE, Klughammer J, Slyper M, Waldman J, Jané-Valbuena J, Rozenblatt-Rosen O, Regev A; IMAXT Consortium, Church GM***+, Marblestone AH***, Boyden ES***+ (2021) Expansion Sequencing: Spatially Precise In Situ Transcriptomics in Intact Biological Systems, Science 371(6528):eaax2656. (* equal contribution, ** key contributions to early stages of project, *** equal contribution, +co-corresponding authors)

Targeted Expansion Sequencing Protocols

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expansion microscopy, in situ sequencing, expansion sequencing, targeted ExSeq, ExSeq, spatial transcriptomics, spatial omics, spatially resolved transcriptomics

protocol ,

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Targeted Expansion Sequencing Protocols

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This protocol has some overlap with the hybridization steps of Expansion FISH (Chen*, Wassie*, et al. (2016)), detailed in more depth in a published Expansion FISH protocol (along with other expansion microscopy protocols) with detailed debugging advice (Asano*, Gao*, Wassie* et al. (2018)). Prospective users are encouraged to review that protocol as well, as it overlaps partly with steps 8, 10, and 14.

A typical gel can fit in a PCR tube or a microcentrifuge tube. The explicit volumes listed in the proocol are for 200 µL, sufficient to fill a PCR tube, and easy to scale up to 300-600 µL, typically enough for samples in microcentrifuge tubes. We note that the volumes can be decreased, as long as the gel is completely submerged. For small gels, 50-100 µL in PCR tubes is sufficient for the hybridization, ligation, and rolling circle amplification steps, which can help save on the cost of reagents.

For washes, the volume should be significantly larger than the volume of the gel. Our suggested volumes are 200 µL for PCR tubes, and 500-1000 µL for microcentrifuge tubes.

Four temperatures are used in this protocol: **4 °C** , **Room temperature** , **30 °C** , **37 °C** . We recommend the use of dedicated, RNAse-free incubators/fridges (i.e., not used for any bacterial or yeast work) with pre-heated/cooled metal tube racks (often called "cold blocks"), or a thermocycler for temperature control.

All reagents used should be RNAse-free. We have provided specific product numbers in the materials section. Of particular note, "water" always refers to UltraPure Water (nuclease-free).

If additional SplintR Ligase Buffer is needed, note that 10X T4 DNA Ligase Buffer (NEB B0202S) is identical to 10X SplintR Ligase Buffer.

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MATERIALS

☒ SplintR Ligase - 6,250 units **New England Biolabs Catalog #M0375L**

☒ Deoxynucleotide (dNTP) Solution Mix **New England Biolabs Catalog #N0447S**

☒ DMSO, Anhydrous **Thermo Fisher Catalog #D12345**

☒ PBS - Phosphate-Buffered Saline (10X) pH 7.4 RNase-free **Thermo Fisher Scientific Catalog #AM9624**

☒ SSC (20X) RNase-free **Thermo Fisher Scientific Catalog #AM9770**

☒ Formamide (Deionized) **Thermo Fisher Scientific Catalog #AM9342**

☒ Triton X-100 **Sigma Aldrich Catalog #T8787-50ML**

☒ UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #10977023**

☒ Aminoallyl-dUTP Solution (50 mM) **Thermo Fisher Scientific Catalog #R1101**

☒ BS(PEG)9 (PEGylated bis(sulfosuccinimidyl)suberate) **Thermo Fisher Scientific Catalog #21582**

☒ T4 DNA Ligase Reaction Buffer **New England Biolabs Catalog #B0202S**

☒ φ29 DNA Polymerase (High Concentration) **Enzymatics Catalog #P7020-HC-L**

☒ UltraPure™ 1M Tris-HCl pH 8.0 **Thermo Fisher Scientific Catalog #15568025**

☒ DAPI Solution (1 mg/mL) **Thermo Fisher Scientific Catalog #62248**

Custom oligonucleotides to order are: (1) the padlock probes (described in the probe generation protocol); (2) the rolling circle amplification primer (described in Step 5); (3) the amplicon detection probe (described in Step 6).

Please carefully read all safety datasheets for all reagents used in the protocol, and perform all steps in accordance with relevant guidelines.

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IP Restrictions.

Prepare stock solutions/reagents as described in the section Preparation of Stock Solutions (Steps 1-3), and Preparation of Oligonucleotide Solutions (Steps 4-6).

The assumed input to the protocol is a sample processed by by a Targeted ExSeq Tissue Preparation, as described in Step 7.

Preparation of Stock Solutions 5m

1 Common Stock Solutions 5m

1. 1X PBS
2. 2X SSC (Saline-Sodium Citrate) buffer
3. Wash buffer: 2X SSC, 20% formamide (v/v) in water
4. Wash-10 buffer: 2X SSC, 10% formamide (v/v) in water
5. Stripping solution: 80% formamide (v/v), 0.01% Triton X-100 (v/v) in water

Suggested volumes to prepare: >10 mL. All buffers can be stored at **Room temperature** . Wash buffer should be used within one month of preparation.

2 4 mM aminoallyl dUTP (aadUTP) 5m

Prepare a 100 µL aliquot of 4 mM aminoallyl dUTP and store at **-20 °C** .

3 250 mM BS(PEG)9 10m

Upon receipt of BS(PEG)9, store in **-20 °C** freezer in original packing.

To aliquot for use: allow BS(PEG)9 vial to warm up to room temperature completely to avoid condensation. Add 465 µL anhydrous DMSO, vortex, and aliquot ~20 µL volumes into PCR tubes.

Store PCR tubes at **-20 °C** in a Parafilm-sealed 50 mL tube containing Drierite.

BS(PEG)9 aliquots should be used within one month from preparation.

Preparation of Oligonucleotide Solutions

4 Pooled Padlock Probes

A more complete discussion of the process of pooling padlock probes is in the probe generation protocol. Briefly, padlock probes should be pooled together such that the concentration of each individual padlock probe is the same, and that the total probe concentration is maximized, typically 200 µM.

We use the per-probe concentration as a convenient metric when calculating dilutions.

As an example, if 40 genes are being interrogated with 16 padlocks/gene, the probes should be pooled together to have a total concentration of 200 µM, with the concentration per probe being 200 µM/(40

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genes * 16 probes/gene) = 312.5 nM per probe.

5 100 µM Rolling Circle Amplification (RCA) Primer

The sequence of the RCA primer is TCT TCA GCG TTC CCG A*G*A, where * denotes phosphorothioate backbone modification. This modification can be included by all major DNA synthesis companies.

Resuspend lyophilized DNA in water to prepare a 100 µM stock solution. Prepare a ~100 µL working stock solution aliquot.

Store RCA primer at **-20 °C** .

6 100 µM Amplicon Detection Oligonucleotide Probe

The sequence of the amplicon detection oligo is /5Cy3/TCTCGGGAACGCTGAAGA, where /5Cy3/ denotes a 5' Cy3 dye. This dye can be replaced with Alexa 546, or any other dye that works well for your imaging setup. Due to the dye-modification, this oligo typically requires HPLC purification.

Resuspend lyophilized DNA in water to prepare a 100 µM stock solution. Prepare a ~25 µL working stock solution aliquot.

Store amplicon detection oligo at **-20 °C** .

Preparation of Samples

7 Initial Preparation of Samples

We assume that samples have been prepared as described by an appropriate Targeted ExSeq Tissue Preparation protocol. In particular, we assume that the total sample thickness after re-embedding and passivation is <350 µm. Thicker samples will require additional optimization.

We assume that the gels have been trimmed and cut to the appropriate size, and are currently stored in 1X PBS in PCR tubes or in microcentrifuge tubes.

See note in Guidelines on volumes. Briefly, volumes described below are 200 µL unless noted otherwise, appropriate for samples in PCR tubes. For all non-wash steps for samples in PCR tubes, volumes can be scaled down to 50-100 µL, as long as the gel is fully submerged. Washes in PCR tubes should remain at 200 µL. For samples in microcentrifuge tubes, recipes can be scaled up appropriately to completely submerge the gel, typically 400-600 µL. Washes in microcentrifuge tubes should be 500-1000 µL in volume.

Since multiple samples are typically processed in parallel, we use the term gels in the following steps. Note that each gel should be processed in its own independent tube.

Padlock Probe Hybridization 1d

8 Padlock Probe Hybridization 1d

The first step of library preparation is to hybridize padlock probes to the RNA transcripts of interest within the expanded samples.

8.1 Pre-hybridization 30m

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Pre-hybridize gels by washing with wash buffer for **00:30:00** at **Room temperature** .

8.2 Preparation of Hybridization Buffer

15m

During the pre-hybridization step, prepare the hybridization buffer. The hybridization buffer consists of the oligonucleotide padlock probes in 2X SSC, 20% formamide.

The concentration of the pooled padlock probes can be variable. We have observed increasing yield with increased per-probe concentration. However, in experiments with a very large number of probes, the per-probe concentration will remain low, due to the total number of probes in solutions. We suggest 100 nM per probe as a starting point for optimization.

Hybridization Mix:

Solution	Stock Concentration	Volume	Final Concentration
SSC buffer	20X	20 µL	2X
Formamide	100%	40 µL	20%
Pooled Padlock Probes	X nM per probe	x µL	variable; suggested 100 nM per probe
Water		(140 - x) µL	
Total		200 µL	

8.3 Padlock Probe Hybridization

1d

Incubate gels with hybridization mix for **Overnight** at **37 °C** .

8.4 Wash Buffer Washes

1h 30m

Wash gels with wash buffer for **00:30:00 x 3 times** at **37 °C** .

For concentrated probe libraries (>100 uM final total probe concentration), this can be increased to 4-5 washes.

8.5 PBS Wash

30m

Wash gels with 1X PBS for **00:30:00** at **37 °C** .

Proceed directly to Padlock Probe Ligation.

Padlock Probe Ligation 13h

9 Enzymatic Ligation of Padlock Probes

13h

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In this step of the protocol, the hybridized padlock probes are enzymatically ligated by SplintR Ligase, forming circular DNA molecules that can subsequently be amplified by rolling circle amplification. After ligation, the padlock probes are topologically intertwined with the RNA transcripts, as the homology region is 32 bp in length.

Of particular importance here is the pre-ligation step, in which SplintR ligase diffuses into the sample at **4 °C** with minimal ligation activity. We found that both extended pre-ligation and pre-amplification steps with SplintR ligase and Phi29 (respectively) are needed to generate amplicons uniformly throughout the volume of the tissue, without any Z-dependence.

9.1 Buffer Pre-equilibration

30m

Wash the gels with 1X SplintR Ligase Buffer for **00:30:00** at **Room temperature** .

9.2 Pre-ligation

6h

Prepare ligation mix at **4 °C** (on a cold block or on ice).

Ligation mix:

Reagent	Stock Concentration	Volume	Final Concentration
Water		170 µL	
SplintR Ligase Buffer	10X	20 µL	1X
SplintR Ligase	25 U/µL	10 µL	1.25 U/µL
Total		**200 µL**	

Incubate gels with pre-ligation mix for **06:00:00** at **4 °C** .

Note: in our testing, the duration of the pre-ligation step may potentially be shortened to three hours, or extended to overnight without adverse effects. We recommend six hours as a starting point for further optimization.

--- Possible Pause Point ---

The pre-ligation step can be run overnight.

9.3 Enzymatic Ligation

6h

Prepare a fresh volume of ligation mix (described in previous substep). Volume can be scaled as described above.

Incubate gels with ligation mix for **06:00:00** at **37 °C** .

Note: in our testing, ligation can be performed overnight if necessary. We recommend six hours, to minimize RNA degradation.

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--- Possible Pause Point ---

The ligation step can be run overnight.

9.4 Ligation Termination

30m

Wash the gels with 2X SSC buffer for **00:30:00** at **Room temperature** .

Proceed directly to RCA Primer Hybridization.

RCA Primer Hybridization 15m

10 RCA Primer Hybridization

3h

In this step, we hybridize a universal primer for rolling circle amplification to the constant sequence of the backbone of the padlock probe. The RCA primer has phosphorothioate modifications to prevent degradation by the strong 3'->5' exonuclease activity of Phi29.

10.1 Pre-hybridization

15m

Pre-hybridize gels by washing with wash buffer for **00:15:00** at **Room temperature** .

10.2 RCA Primer Hybridization

2h

Prepare the RCA primer hybridization mix by diluting the 100 µM RCA primer stock by 1:200 in wash buffer, forming a 500 nM RCA primer solution.

Incubate gels in RCA primer hybridization mix for **02:00:00** at **37 °C** .

Note: in our testing, RCA primer hybridization can be run overnight if necessary. We recommend two hours, to minimize RNA degradation.

--- Possible Pause Point ---

The RCA primer hybridization can be run overnight.

10.3 Wash Buffer Wash

30m

Wash gels with wash buffer for **00:30:00** at **37 °C** .

10.4 PBS Wash

15m

Wash gels with 1X PBS for **00:15:00** at **37 °C** .

Proceed directly to Rolling Circle Amplification (RCA).

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11 Rolling Circle Amplification

1d

In this step, the ligated padlock probes are amplified using rolling circle amplification (RCA), forming amplicons (colloquially called "RCA colonies" or "rolonies").

Similar to the ligation step, we have an extended pre-amplification step, in which the Phi29 enzyme diffuses into the sample at **4 °C** . During RCA, aminoallyl dUTP is incorporated into the amplicons, enabling subsequent crosslinking by BS(PEG)9.

11.1 Buffer Pre-equilibration

15m

Wash the gels with 1X Phi29 Buffer for **00:15:00** at **Room temperature** .

11.2 Pre-amplification

6h

Prepare pre-amplification mix at **4 °C** (on a cold block or on ice).

Pre-amplification mix:

Reagent	Stock Concentration	Volume	Final Concentration
Water		178 µL	
Phi29 Buffer	10X	20 µL	1X
Phi29 DNA Polymerase	100 U/µL	2 µL	1 U/µL
Total		**200 µL**	

Incubate gels with pre-amplification mix for **06:00:00** at **4 °C** .

Note: in our testing, the duration of the pre-amplification step may potentially be shortened to three hours, or extended to overnight without adverse effects. We recommend six hours as a starting point for further optimization as we have used this duration most often.

--- Possible Pause Point ---

The pre-amplification step can be run overnight.

11.3 Rolling Circle Amplification

1d

Prepare RCA mix at **4 °C** (on a cold block or on ice).

RCA mix:

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A	B	C	D
Reagent	**Stock Concentration**	**Volume**	**Final Concentration**
Water		171 µL	
Phi29 Buffer	10X	20 µL	1X
dNTP	10 mM each nucleotide	5 µL	250 µM
Aminoallyl dUTP (aadUTP)	4 mM	2 µL	40 µM
Phi29 DNA Polymerase	100 U/µL	2 µL	1 U/µL
Total		**200 µL**	

Incubate gels with RCA mix for **Overnight** at **30 °C** .

--- Pause Point ---

The RCA reaction runs overnight.

11.4 PBS Wash

30m

Terminate the RCA by washing the sample with 1X PBS for **00:30:00** at **Room temperature** .

Proceed directly to BS(PEG)9 Cross-Linking

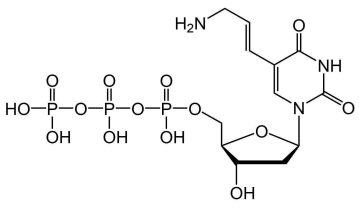
BS(PEG)9 Cross-Linking 2h 45m

12 BS(PEG)9 Cross-Linking

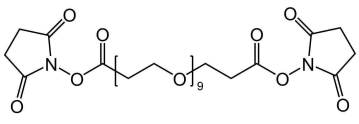
2h 45m

To prevent amplicon movement across multiple rounds of in situ sequencing, amplicons are covalently anchored to themselves, resulting in a cross-linked DNA amplicon that is enmeshed with the expansion gel.

To accomplish this, aminoallyl dUTP (aadUTP) is included in the RCA mix. In this step, the NHS esters of BS(PEG)9 react with the primary amines of the allylamine groups, crosslinking the amplicon to itself. The structures of aadUTP and BS(PEG)9 are shown below.



Aminoallyl dUTP (aadUTP)



BS(PEG)9

12.1 Amplicon Cross-linking

2h

Thaw one aliquot of reconstituted 250 mM BS(PEG)9 at room temperature.

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Prepare crosslinking mix:

Reagent	Stock Concentration	Volume	Final Concentration
PBS	1X	196 µL	~1X
BS(PEG)9	250 mM	4 µL	5 mM
Total		**200 µL**	

Incubate gels with crosslinking mix for **02:00:00** at **Room temperature** .

12.2 Tris Wash

15m

Quench reaction by washing gels with 1 M Tris pH 8 for **00:15:00** at **Room temperature** .

12.3 PBS Wash

30m

Wash gels with 1X PBS for **00:30:00** at **Room temperature** .

13 [OPTIONAL] Long Term Storage

At this point, the library preparation process is complete, and the amplicons are stable.

For long-term storage, replace the 1X PBS solution once more, then store gels in well-sealed containers at **4 °C** for up to several months.

Universal Amplicon Detection Hybridization 3h 35m

14 Amplicon Detection Hybridization

3h 35m

A rapid quality control assay for the library preparation process is to visualize all amplicons within the specimen by hybridizing a fluorophore-labeled oligonucleotide probe to the amplicon constant region, thereby labeling all amplicons for imaging.

14.1 Pre-hybridization

20m

Wash gels with Wash-10 buffer for **00:20:00** at **Room temperature** .

14.2 Detection Probe Hybridization

1h

Prepare the amplicon detection probe hybridization mix by diluting the 100 µM amplicon detection probe stock by 1:1000 in Wash-10 buffer, forming a 100 nM amplicon detection probe solution.

Incubate gels in amplicon detection probe solution for **01:00:00** at **37 °C** .

Wash-10 Wash

20m

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14.3

Wash gels with Wash-10 buffer for **00:20:00** at **37 °C** .

20m

14.4 PBS Wash

Wash gels with 1X PBS for **00:20:00** at **37 °C** .

25m

14.5 DAPI Staining

Stain gels with DAPI in 1X PBS. Recipe for 1 mL DAPI solution:

Reagent	Stock Concentration	Volume	Final Concentration
PBS	1X	999 µL	1X
DAPI	1 mg/mL	1 µL	1 mg/L
Total		**1000 µL**	

Stain gel with DAPI solution for **00:15:00** at **Room temperature** .

Wash with 1X PBS for **00:10:00** at **Room temperature** .

Remove 1X PBS thoroughly to prevent gel movement during imaging.

14.6 Imaging

After PBS wash, remove excess PBS and use a clean paintbrush to transfer gel to a glass-bottom 6-well or 24-well plate for imaging.

Image as is appropriate for the dye used to visualize amplicons. Individual amplicons should be visible at 10X and higher magnification air-objectives, with significantly higher image quality with 20X and higher magnification, and high NA, water/oil immersion objectives. Our standard objective for expansion microscopy is the Nikon 40X Long Working Distance, Water Immersion, NA 1.15 objective (CFI ApoLWD Lambda S 40XC WI).

30m

14.7 Stripping Detection Oligo

Transfer the gel from the imaging plate to an appropriate smaller container, i.e. PCR tube or microcentrifuge tube.

Heat an appropriate amount of stripping solution to **80 °C** . The stripping solution can be heated in a thermocycler. The total volume pre-heated should be enough for three full washes, i.e. 600 µL for gels in PCR tubes.

Wash the sample with pre-heated stripping solution for **00:10:00 x 3 times** . For each wash, use pre-heated stripping solution, but keep the sample at **Room temperature** .

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14.8 PBS Washes and Storage

20m

Wash gels with 1X PBS for **00:10:00 x 2 times** at **Room temperature** . Then, store as described in Step 13.

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