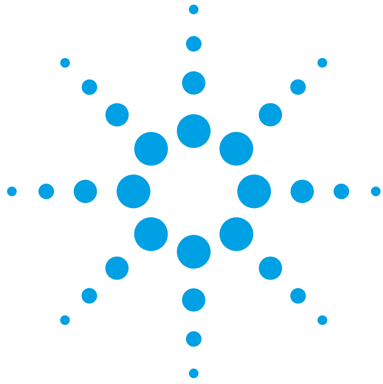

Gene Expression FFPE Workflow

Quick Start Guide

For Research Use Only. Not for use in diagnostic procedures.

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This protocol supports 8-pack microarray format only.

Sigma TransPlex WTA Kit Overview

The TransPlex Whole Transcriptome Amplification (WTA) kit allows for rapid amplification of total RNA from formalin-fixed, paraffin embedded (FFPE) samples in less than 4 hours without 3'-bias.

Agilent Technologies

The WTA kit involves two steps. First, total RNA is reverse-transcribed with a WTA Polymerase using non-complementary primers composed of quasi-random 3' end and a universal 5' end. Then, the resulting Omniplex cDNA library, composed of overlapping 100 to 1000 base fragments, is PCR-amplified to produce microgram quantities of WTA products for downstream applications such as qPCR and microarray analyses.

NOTE Sigma makes two different WTA kits: WTA1 and WTA2. This protocol uses the WTA1 kit.

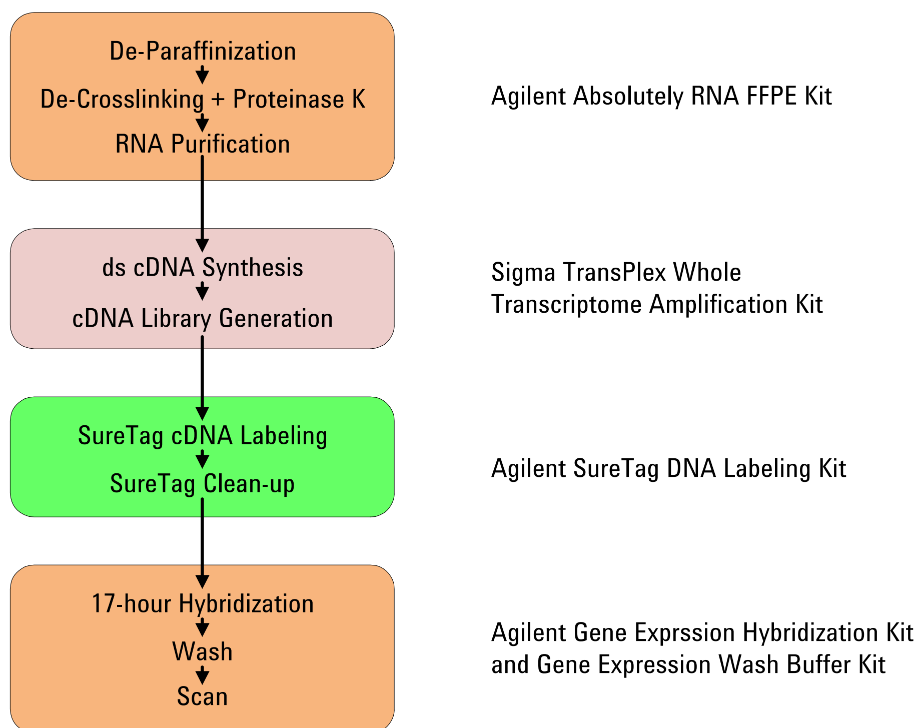

Figure 1 Various kits and steps involved in the analysis of FFPE samples with the Agilent gene expression microarray workflow.

Before you Begin

Make sure you have the materials listed in this section.

Required Equipment

Table 1 Required Equipment

Description	Vendor and part number
Agilent Microarray Scanner	Agilent p/n G4900DA, G2565CA or G2565BA
Hybridization Chamber, stainless	Agilent p/n G2534A
Hybridization gasket slides 8 microarrays/slide, 5 slides/box	Agilent p/n G2534-60014
Hybridization oven; temperature set at 65°C	Agilent p/n G2545A
Hybridization oven rotator for Agilent Microarray Hybridization Chambers	Agilent p/n G2530-60029
Nuclease-free 1.5 mL microfuge tube	Ambion p/n 12400 or equivalent
Magnetic stir bar (×2)	Corning p/n 401435 or equivalent
Magnetic stir plate (×2)	Corning p/n 6795-410 or equivalent
Microcentrifuge	Eppendorf p/n 5417R or equivalent
NanoDrop ND-1000 UV-VIS spectrophotometer	NanoDrop p/n ND-1000 or equivalent
Slide-staining dish, with slide rack (×3)	Thermo Shandon p/n 121 or equivalent
Circulating water baths of heat blocks set to 37°C, 40°C, 60°C, 65°C, and 80°C	
96-well PCR plate	
Clean forceps	

Table 1 Required Equipment (continued)

Description	Vendor and part number
Ice bucket	
Pipetman micropipettors, (P-10, P-20, P-200, P-1000) or equivalent	
Powder-free gloves	
Sterile, nuclease-free aerosol barrier pipette tips	
Vortex mixer	
Timer	
Nitrogen purge box for slide storage	

Required Reagents

Table 2 Required Reagents

Description	Vendor and part number
SureTag DNA Labeling Kit	Agilent p/n 5190-3400
Gene Expression Hybridization Kit *or* Hi-RPM Gene Expression Hybridization Kit, Large Volume	Agilent p/n 5188-5242 Agilent p/n 5190-0404
Gene Expression Wash Buffer pack	Agilent p/n 5188-5327
100% Ethanol	Amresco p/n E193
TITANIUM Taq DNA Polymerase	Clontech p/n 639208
DNase/RNase-free distilled water	Invitrogen p/n 10977-015
1×TE (pH 8.0), Molecular grade	Promega p/n V6231
QIAquick PCR Purification Kit	Qiagen p/n 28106
Sulfolane	Sigma p/n T22209
Transplex Whole Transcriptome Amplification kit	Sigma p/n WTA1-50RXN

Table 2 Required Reagents

Description	Vendor and part number
Absolutely RNA FFPE Kit	Agilent p/n 400809
Milli-Q water or equivalent	

Optional Equipment/Reagents

Table 3 Optional Equipment/Reagents

Description	Vendor and part number
2100 Bioanalyzer	Agilent p/n G2938A
RNA 6000 Nano Assay Kit (RNA Series II Kit)	Agilent p/n 5067-1511
Stabilization and Drying Solution	Agilent p/n 5185-5979
Ozone-Barrier Slide Cover	Agilent p/n G2505-60550
Slide box	Corning p/n 07201629
Acetonitrile	Sigma p/n 271004-1L
Vacuum concentrator	Savant SpeedVac p/n SPD111V, Thermo Scientific p/n DNA120-115, or equivalent

Procedure

Step 1. Isolate RNA

1 Extract RNA from FFPE blocks. Follow the instructions in the user manual for the Absolutely RNA FFPE Kit, available at http://www.chem.agilent.com/Library/usermanuals/Public/400809.pdf.

   For the Proteinase K Digestion step of the Absolutely RNA FFPE Kit manual, incubate the tubes at 55°C for 18 hours instead of 3 hours.

Step 2. Prepare WTA Library

1 Thaw the WTA Library Synthesis Buffer and WTA Library Stabilization Solution on ice and mix on a vortex mixer. If either solution has a precipitate, briefly heat at 37°C and mix the tube(s) on a vortex mixer until the precipitate is gone.

2 Add WTA Nuclease-Free Water to 25 to 300 ng of FFPE-extracted RNA to get a total volume of 19 µL (use tubes/strips/plates that will fit in a PCR thermal cycler).

3 Prepare the WTA Library Preparation Mix:

Table 4 WTA Library Preparation Mix

Components	Per reaction (µL)	Per slide (µL) (including excess)
WTA Library Synthesis Buffer	2.5	25
WTA Library Stabilization Solution	2.5	25
Final Volume	5	50

4 Add 5 µL of the WTA Library Preparation Mix to FFPE-extracted RNA for a total volume of 24 µL.

5 Mix samples well by pipetting and incubate at 70°C for 5 minutes.

6 Cool reaction on ice and briefly centrifuge liquid to bottom of PCR plate.

7 Add 1 µL of WTA Library Synthesis Enzyme to each sample for a total volume of 25 µL and mix well by pipetting.

8 Place PCR plate in thermal cycler and incubate as follows:
   - 24°C for 15 minutes
   - 42°C for 2 hours
   - 95°C for 5 minutes
   - 4°C - hold

9 Cool reaction on ice and briefly centrifuge PCR plate.

Step 3. Amplify WTA Library

1 Immediately prior to use, gently mix the components listed in Table 5 for the WTA Amplification Mix by adding in the order indicated, and keep on ice.

NOTE TITANIUM Taq DNA Polymerase is an enzyme, which needs to be kept on ice and added to the WTA Amplification Mix just before starting the reactions.

Table 5 WTA Amplification Mix

Components	Per reaction (µL)	Per slide (µL) (including excess)
WTA Nuclease-Free Water	300	3000
WTA Amplification Master Mix	37.5	375
WTA dNTP Mix	7.5	75
TITANIUM Taq DNA Polymerase	5	50
Final Volume	350	3500

2 Divide the library generated in “Step 2. Prepare WTA Library” (25 µL) into 5 equivalent 5 µL aliquots in the wells of a PCR plate.

   Depending on the tissue and the age of the FFPE block, the amount of yield from 1 aliquot can vary. Typically, 1 or 2 aliquots is enough for the SureTag labeling step.

3 Add 70 µL of the WTA Amplification Mix (Table 5) to each library aliquot and mix well by gently pipetting up and down. The final reaction volume should be 75 µL.

4 Place PCR plate in thermal cycler and cycle as follows:

   - 95°C for 3 minutes
   - 94°C for 20 seconds, 65°C for 5 minutes, for 22 cycles
   - 4°C - Hold

5 Cool reaction on ice and briefly centrifuge PCR plate.

The amplified library aliquots must be purified and pooled before further analysis. The purified and pooled amplified library can be stored at -20°C.

Step 4. Purify WTA products using the QIAquick PCR Purification Kit

All centrifuge steps are at 13,000 rpm (approximately 17,900 x g) in a conventional tabletop microcentrifuge.

Use the reagents from the QIAquick PCR Purification Kit.

NOTE For best results, purify individual amplified aliquots separately, then pool them together.

1. Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix (for example, 375 µL of Buffer PB to 75 µL PCR product).
2. Put a QIAquick Spin Column in provided 2 mL collection tube.
3. To bind DNA to the column, apply the sample to the QIAquick Spin Column and spin in a centrifuge for 30 to 60 seconds.
4. Discard the flow-through. Place the QIAquick Spin Column back into the same tube.
5. To wash, add 0.75 mL Buffer PE (concentrate) to the QIAquick Spin Column and centrifuge for 30 to 60 seconds.
6. Discard flow-through and place the QIAquick Spin Column back in the same tube. Centrifuge the column for an additional 1 minute.
7. Place QIAquick Spin Column in a clean Nuclease-free 1.5 mL microfuge tube.
8. To elute the DNA, add 50 µL of DNase/RNase-free distilled water to the center of the QIAquick membrane, let the column stand for 1 minute, and then spin the column in a centrifuge for 1 minute.
9. Move to ice.
10. Quantitate with NanoDrop using 1.5 µL of the eluted DNA.

Step 5. Label with SureTag DNA Labeling Kit

1 For an 8-pack microarray hybridization, prepare a tube that contains 1.8 µg of cDNA in a final volume of 26 µL.

   If the 1.8 µg of cDNA is in a volume greater than 26 µL, use a vacuum concentrator to concentrate the sample until the volume is 26 µL.

2 Add 5 µL of Random Primer to the tube for a final sample volume of 31 µL.

3 Incubate at 95°C for 5 minutes.

4 Transfer samples to ice and incubate on ice for 5 minutes.

5 Spin in a microcentrifuge for 1 minute at 6000 × g to drive contents off the walls and lid.

6 Mix the components in Table 6 to prepare Cyanine 3 Labeling Master Mix:

Table 6 Cyanine 3 Labeling Master Mix

Components	Per reaction (µL)	Per slide (µL) (including excess)
5× Reaction Buffer	10	100
10× dNTPs	5	50
Cyanine 3-dUTP	3	30
Exo (-) Klenow	1	10
Final Volume	19	190

7 Add 19 µL of Cyanine 3 Labeling Master Mix to each tube that contains the 31 µL of cDNA for a total volume of 50 µL. Mix well by gently pipetting up and down.

8 Incubate tubes at 37°C for 2 hours.

9 Transfer samples to 65°C and incubate for 10 minutes to inactivate enzyme.

10 Transfer samples to ice and incubate on ice for 3 minutes.

11 Spin in a microcentrifuge for 1 minute at 6000 × g to drive contents off the walls and lid.

Store labeled cDNA on ice until excess dye is removed using the columns included in the SureTag DNA Labeling Kit.

Step 6. Purify SureTag-labeled cDNA

The Purification Column used in this step is included in the SureTag DNA Labeling Kit.

1. Spin the labeled cDNA samples in a centrifuge for 1 minute at 6,000 × g to drive the contents off the walls and lid.
2. Add 430 µL of 1×TE (pH 8.0) to each reaction tube.
3. For each cDNA sample to be purified, place a reaction Purification Column into a 2-mL collection tube and label the Purification Column appropriately. Load each labeled cDNA onto a Purification Column.
4. Cover the Purification Column with a cap and spin for 10 minutes at 14,000 × g in a microcentrifuge at room temperature. Discard the flow-through and place the Purification Columns back in the collection tubes.
5. Add 480 µL of 1×TE (pH 8.0) to each Purification Column. Spin for 10 minutes at 14,000 × g in a microcentrifuge at room temperature. Discard the flow-through.
6. Invert the filter into a fresh Nuclease-free 1.5 mL microfuge tube that has been appropriately labeled. Spin for 1 minute at 1,000 × g in a microcentrifuge at room temperature to collect purified sample.

   The volume per sample will be approximately 20 to 32 µL.
7. Concentrate the labeled cDNA sample to dryness and resuspend in 21.5 mL of 1×TE (pH 8.0).
8. Use a NanoDrop to calculate the degree of labeling, specific activity, and yield of 1.5 µL of the eluted sample.

$$\text{Degree of labeling} = \frac{340 \times \text{pmol per } \mu\text{L dye}}{\text{ng per } \mu\text{L cDNA} \times 1000} \times 100\%$$

$$\text{Specific Activity} = \frac{\text{pmol per } \mu\text{L of dye}}{\mu\text{g per } \mu\text{L cDNA}}$$

$$\text{Yield } (\mu\text{g}) = \frac{\text{DNA Concentration (ng/}\mu\text{L)} \cdot \text{Sample Volume } (\mu\text{L})}{1000 \text{ ng/}\mu\text{g}}$$

Step 7. Prepare labeled cDNA for hybridization

1 Prepare 10× Gene Expression Blocking Agent:
   a Add DNase/RNase-free distilled water (500 µL for Gene Expression Hybridization Kit or 1250 µL for Hi-RPM Gene Expression Hybridization Kit, Large Volume) to the vial of lyophilized 10× Gene Expression Blocking Agent.
   b Mix briefly on a vortex mixer and leave at room temperature for 60 minutes to reconstitute sample before use or storage.
2 Equilibrate water baths or heat blocks to 95°C.
3 Mix the components in Table 7 to prepare the Hybridization Master Mix:

Table 7 Hybridization Master Mix for 8-pack microarrays

Components	Per reaction (µL)	Per slide (µL) (including excess)
10× Gene Expression Blocking Agent	5	50
2× Hi-RPM Hybridization Buffer	25	250
Final Volume	30	300

4 Add 30 µL of the Hybridization Master Mix to the 20 µL of Cyanine-labeled cDNA (from step 8 on page 12) for a total volume of 50 µL.
5 Incubate at 95°C for 3 minutes, then place on ice.
6 Spin samples briefly in a microcentrifuge to drive contents off the walls and lid.
7 Dispense 40 µL of sample onto each 8×60K microarray.
8 Hybridize at 65°C for 17 hours at 20 RPM.
9 Use standard Agilent Gene Expression wash and scan conditions.

   Refer to the *One-color Microarray-based Gene Expression Analysis (Low Input Quick Amp Labeling) Protocol* (p/n G4140-90040).

Reference

Kit Contents

Table 8 Transplex Whole Transcriptome Amplification kit

Component
WTA Library Synthesis Buffer
WTA Library Stabilization Solution
WTA Amplification Master Mix
WTA Library Synthesis Enzyme
WTA Nuclease-Free Water
WTA dNTP Mix

Table 9 QIAquick PCR Purification Kit

Component
QIAquick Spin Column
Buffer PB
Buffer PE (concentrate)
Buffer EB
pH Indicator I
Collection Tube
Loading Dye

Table 10 SureTag DNA Labeling Kit

Component
10× Restriction Enzyme Buffer
BSA
Alu I
Rsa I
Purification Column
Nuclease-Free Water
Exo (-) Klenow
5× Reaction Buffer
Cyanine 5-dUTP
Cyanine 3-dUTP
10× dNTPs
Random Primer

Table 11 Gene Expression Hybridization Kit

Component
10× Gene Expression Blocking Agent
25× Fragmentation Buffer
2× Hi-RPM Hybridization Buffer

Table 12 Gene Expression Wash Buffer pack

Component
Gene Expression Wash Buffer 1
Gene Expression Wash Buffer 2
Triton X-102 (10%)

Microarray QC Metrics for FFPE samples

These metrics are only appropriate for samples analyzed with Agilent Gene Expression microarrays by following the standard operational procedures provided in this protocol. These metrics are exported to a table in the Feature Extraction QC report.

The QC metrics can be used to assess the relative data quality across a set of microarrays in an experiment. In some cases, they can indicate potential processing errors that may have occurred or suggest that the data from particular microarrays might be compromised.

Many factors can influence the range of these metrics including the biological sample source, quality of starting FFPE samples, experimental processing, scanner sensitivity and image processing. The value guidelines presented below represent the thresholds that Agilent has observed when analyzing FFPE samples using this protocol.

Table 13 QC metric thresholds

	FFPE Samples
AnyColorPrcntFeatNonUnifOL	< 1
gNegCtrlAveNetSig	< 40
gNegCtrlAveBGSubSig	-10 to 5
gNegCtrlSDevBGSubSig	< 10
gSpatialDetrendRMSFit	< 15
gNonCntrlMedCVProcSig	0 to 8

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In This Book

The *Quick Start Guide* presents overview instructions to process FFPE RNA samples.

What's New in 2.0

- SureTag DNA Labeling Kit replaces Agilent gDNA ULS Labeling Kit.
- Support for 8×60K microarray format.

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