# GeneChip® Expression 3’-Amplification Reagents for IVT Labeling

P/N 900449 (30 eukaryotic reactions)

*In vitro* transcription with MEGAscript™.

## INTRODUCTION

The GeneChip® IVT Labeling Kit is optimized specifically for producing amplified and biotinylated targets to hybridize to eukaryotic GeneChip brand arrays for expression analysis. Each kit contains sufficient reagents for completing 30 *in vitro* transcription reactions.

The template DNA for this IVT reaction is typically double-stranded cDNA containing the T7 promoter sequence. In GeneChip target labeling experiments, the T7 promoter sequence is incorporated into the cDNA template by using a T7-Oligo(dT) Promoter Primer in the initial reverse transcription reaction (described in the *GeneChip® Expression Analysis Technical Manual*, available at www.affymetrix.com). Sufficient cDNA template can be obtained using one of the two protocols from high-quality starting materials:

- 1 to 15 µg of sample total RNA, or 0.2 to 2 µg of mRNA, using the One-Cycle cDNA Synthesis Procedure
- 10 to 100 ng of sample total RNA using the Two-Cycle cDNA Synthesis Procedure

Using this IVT Labeling Kit, over 30 µg of biotinylated cRNA targets are usually generated with the MEGAscript™ T7 Polymerase (**IVT Labeling Enzyme Mix**) in the presence of a mixture of the four natural ribonucleotides and one biotin-conjugated nucleotide analog at optimized ratio (**IVT Labeling NTP Mix**). The chemical structure of the analog is illustrated in Figure 1.

The synthetic analog is efficiently incorporated into the cRNA target during the *in vitro* transcription labeling reaction mediated by T7 RNA polymerase as a pseudouridine reagent. The biotinylated cRNA targets are then purified, fragmented, and hybridized to GeneChip expression arrays.

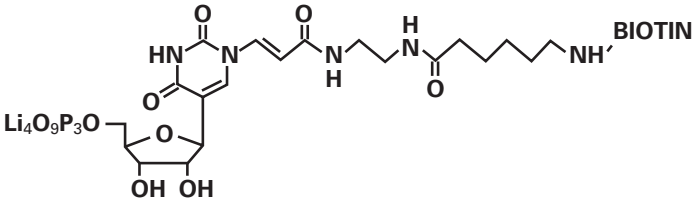


**Figure 1. Structure of the biotin-conjugated nucleotide analog**

A positive control, the **3’- Labeling Control**, is included in the kit and allows users to test the transcriptional activity of the kit components. Using 0.5 µg of the **3’- Labeling Control** as a template, routinely, over 30 µg of biotinylated IVT product is generated after 4 hours of incubation. The amplified, labeled product can be visually inspected on an agarose gel or other electrophoretic system, and a distinct band at approximately 2 Kb should be observed.

**NOTE** Hybridization cocktail preparation and fluidics scripts have been optimized specifically for using this IVT Labeling Kit. Follow the instructions described below for best results.

## CONTENTS

| Volume | Component |
|---|---|
| 120 µL | 10X IVT Labeling Buffer |
| 120 µL | IVT Labeling Enzyme Mix |
| 360 µL | IVT Labeling NTP Mix |
| 10 µL | 3’-Labeling Control (0.5 µg/µL) |
| 910 µL | RNase-free Water |

## STORAGE

Kit should be kept at -20°C in a non-frost-free freezer. Performance of the kit has been shown to be unaffected for up to eight freeze-thaw cycles.

## GENECHIP EUKARYOTIC TARGET LABELING ASSAYS FOR EXPRESSION ANALYSIS

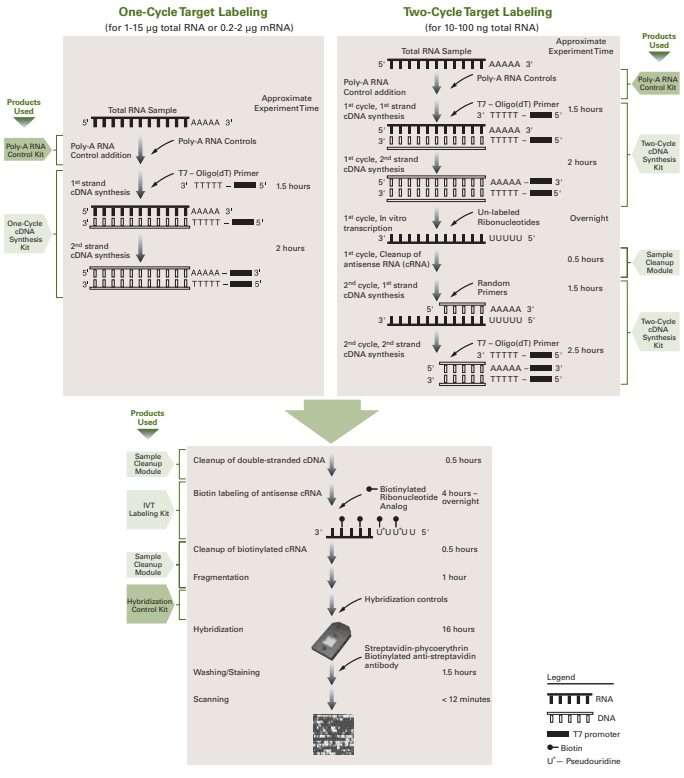


**Figure 2. Schematic illustration of GeneChip® target labeling assays for expression analysis**

## OTHER NECESSARY REAGENTS NOT PROVIDED WITH THE KIT

| Kit name | P/N | Size |
|---|---|---|
| One-Cycle cDNA Synthesis Kit | 900431 | 30 rxns |
| Two-Cycle cDNA Synthesis Kit | 900432 | 30 rxns |
| Sample Cleanup Module | 900371 | 30 rxns |
| Eukaryotic Poly-A RNA Control Kit | 900433 | ~ 100 rxns |
| Hybridization Controls | 900454 and 900457 | 30 rxns and 150 rxns |

## IVT AMPLIFICATION AND LABELING PROCEDURE

**NOTE** This kit is only used for the IVT labeling step for generating biotin-labeled cRNA. For the IVT amplification step using unlabeled ribonucleotides in the First Cycle of the Two-Cycle cDNA Synthesis Procedure, a separate kit is recommended (MEGAscript® T7 Kit, Ambion, Inc.). Use only nuclease-free water, buffers, and pipette tips.

**IMPORTANT** Store all reagents in a -20°C freezer that is not self-defrosting. Prior to use, centrifuge all reagents briefly to ensure that the solution is collected at the bottom of the tube.

**IMPORTANT** The Target Hybridizations and Array Washing protocols have been optimized specifically for this IVT Labeling Protocol. Closely follow the recommendations described below for maximum array performance.

1. Use the following table to determine the amount of cDNA used for each IVT reaction following the cDNA cleanup step.

| Starting Material | Volume of cDNA to use in IVT |
|---|---|
| **Total RNA** | |
| 10 to 100 ng | all (~12 µL) |
| 1.0 to 8.0 µg | all (~12 µL) |
| 8.1 to 15 µg | 6 µL |
| **mRNA** | |
| 0.2 to 2 µg | all (~12 µL) |

2. Transfer the needed amount of template cDNA to RNase-free microfuge tubes and add the following reaction components in the order indicated in the table below. If more than one IVT reaction is to be performed, a master mix can be prepared by multiplying the reagent volumes by the number of reactions. Do not assemble the reaction on ice, since spermidine in the **10X IVT Labeling Buffer** can lead to precipitation of the template cDNA.

| Reagent | Volume |
|---|---|
| Template cDNA* | variable (see table above) |
| RNase-free Water | variable (to give a final reaction volume of 40 µL) |
| 10X IVT Labeling Buffer | 4 µL |
| IVT Labeling NTP Mix | 12 µL |
| IVT Labeling Enzyme Mix | 4 µL |
| **Total Volume** | **40 µL** |

*1 µg of the 3'-Labeling Control can be used in place of the template cDNA sample in this reaction as a positive control for the IVT components in the kit.

3. Carefully mix the reagents and collect the mixture at the bottom of the tube by brief (5 second) microcentrifugation.
4. Incubate at 37°C for 16 hours. To prevent condensation that may result from water bath-style incubators, incubations are best performed in oven incubators for even temperature distribution, or in a thermal cycler.

**NOTE** Overnight IVT reaction time has been shown to maximize the labeled cRNA yield with high-quality array results. Alternatively, if a shorter incubation time (4 hours) is desired, 1 µL (200 units) of cloned T7 RNA polymerase (can be purchased directly from Ambion, P/N 2085) can be added to each reaction and has been shown to produce adequate labeled cRNA yield within 4 hours. The two different incubation protocols generate comparable array results, and users are encouraged to choose the procedure that best fits their experimental schedule and process flow.

5. Store labeled cRNA at -20°C, or -70°C if not purifying immediately. Alternatively, proceed to Cleanup and Quantification of Biotin-Labeled cRNA as described in the *GeneChip Expression Analysis Technical Manual*.

## EUKARYOTIC TARGET HYBRIDIZATION

1. Mix the following for each target, scaling up volumes for hybridization to multiple probe arrays.

**NOTE** A final concentration of 10% DMSO is added in the Hybridization Cocktail for optimal results.

**IMPORTANT** It is imperative that the frozen 20X Hybridization Control Stock is heated to 65°C for 5 minutes to completely resuspend the cRNA before adding to the hybridization cocktail.

| Component | 49 Format/ 64 Format | 100 Format | 400 Format/ 169 Format | Final Concentration |
|---|---|---|---|---|
| Fragmented cRNA** | 15 µg | 10 µg | 5 µg | 0.05 µg/µL |
| Control Oligonucleotide B2 (3 nM) | 5 µL | 3.3 µL | 1.7 µL | 50 pM |
| 20X Eukaryotic Hybridization Controls (*bioB*, *bioC*, *bioD*, *cre*) | 15 µL | 10 µL | 5 µL | 1.5, 5, 25 and 100 pM respectively |
| Herring Sperm DNA (10 mg/mL) | 3 µL | 2µL | 1 µL | 0.1 mg/mL |
| Acetylated BSA (50 mg/mL) | 3 µL | 2 µL | 1 µL | 0.5 mg/mL |
| 2X Hybridization Buffer | 150 µL | 100 µL | 50 µL | 1X |
| DMSO | 30 µL | 20 µL | 10 µL | 10% |
| $H_2O$ | to final volume of 300 µL | to final volume of 200 µL | to final volume of 100 µL | |
| **Final volume** | **300 µL** | **200 µL** | **100 µL** | |

*Please refer to specific probe array package insert for information on array format.

**Please see Section 2, Chapter 1 in the *GeneChip Expression Analysis Technical Manual* for amount of adjusted fragmented cRNA to use when starting from total RNA.

2. Hybridize to expression probe arrays as described in the *Expression Analysis Technical Manual*.

## WASHING AND SCANNING

1. Prepare the SAPE solution and Antibody solution as described in Section 2, Chapter 4 of the *Expression Analysis Technical Manual* using the Antibody Amplification Stain Protocol for Eukaryotic Targets.
2. **For 49 Format and 64 Format arrays with 18 µm features**, use the existing EukGE-WS2v4 or EukGE-WS2v4_450 Fluidics Scripts.

   **For 49 Format and 64 Format arrays with 11 µm features,** download the new EukGE-WS2v5 Fluidics Scripts from www.affymetrix.com. Alternatively, modify the FlexEukGE-WS2v4 or FlexEukGE-WS2v4_450 Fluidics Scripts as follows, and rename the scripts as EukGE-WS2v5 or EukGE-WS2v5_450.

   **For 100 Format arrays**, use the existing Midi-euk2 or Midi-euk2_450 Fluidics Scripts.

| | 49 Format/ 64 Format EukGE-WS**2v5** | 100 Format (Midi) Midi_euk2[a] |
|---|---|---|
| **Post Hyb Wash #1** | 10 cycles of 2 mixes/cycle with Wash Buffer A at 30°C | 10 cycles of 2 mixes/cycle with Wash Buffer A at 30°C |
| **Post Hyb Wash #2** | 6 cycles of 15 mixes/cycle with Wash Buffer B at 50°C | 6 cycles of 15 mixes/cycle with Wash Buffer B at 50°C |
| **Stain** | Stain the probe array for 5 minutes in SAPE solution at 35°C | Stain the probe array for 5 minutes in SAPE solution at 35°C |
| **Post Stain Wash** | 10 cycles of 4 mixes/cycle with Wash Buffer A at 30°C | 10 cycles of 4 mixes/cycle with Wash Buffer A at 30°C |
| **2nd Stain** | Stain the probe array for 5 minutes in antibody solution at 35°C | Stain the probe array for 5 minutes in antibody solution at 35°C |
| **3rd Stain** | Stain the probe array for 5 minutes in SAPE solution at 35°C | Stain the probe array for 5 minutes in SAPE solution at 35°C |
| **Final Wash** | 15 cycles of 4 mixes/cycle with Wash Buffer A at 35°C. The holding temperature is 25°C | 15 cycles of 4 mixes/cycle with Wash Buffer A at 35°C. The holding temperature is 25°C |

- Wash Buffer A = non-stringent wash buffer
- Wash Buffer B = stringent wash buffer

a. Midi_euk2_450 for the FS-450/250

## FUNCTIONAL TESTING AND QUALITY CONTROL

### GeneChip Array Functional Testing

Each lot is functionally tested in the GeneChip expression assay following the recommended protocol.

## SAFETY INFORMATION

An MSDS is available from Affymetrix. The kit components are not believed to present any health hazard. However, the chemical, physical, and toxicological properties of these components have not been thoroughly investigated.

## CONTACT INFORMATION

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