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StemPro® EZChek™ Human Tri-Lineage Multiplex PCR Kit

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User Manual

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Contents and Storage

Kit Configuration

StemPro® EZChek™ Human Tri-Lineage Multiplex PCR Kit includes the following boxes, providing material and reagents for 100 reactions. For a detailed description of the contents, see below and next page.

- PureLink™ Micro-to-Midi™ Total RNA Purification System
- SuperScript™ III First-Strand Synthesis SuperMix
- StemPro® EZChek™ Human Tri-Lineage Primer Mix

Shipping and Storage

The StemPro® EZChek™ Human Tri-Lineage Multiplex PCR Kit is shipped as described below. Upon receipt, store each item as detailed below.

Component	Shipping	Storage
PureLink™ Micro-to-Midi™ Total RNA Purification System	Room temperature	Room temperature
SuperScript™ III First-Strand Synthesis SuperMix	Dry ice	-20°C (non-frost-free)
StemPro® EZChek™ Human Tri-Lineage Primer Mix	Dry ice	-20°C (non-frost-free)

PureLink™ Micro-to-Midi™ Total RNA Purification System

Two boxes of the PureLink™ Micro-to-Midi™ Total RNA Purification System are provided. Components are listed below. Sufficient reagents are provided to perform 100 isolations (50 isolations per box × 2). **Store reagents at room temperature**.

Component	Amount
RNA Lysis Solution	125 ml × 2
Wash Buffer I	50 ml × 2
Wash Buffer II	15 ml × 2
RNase-Free Water	15 ml × 2
RNA Spin Cartridges with collection tubes	50 × 2
RNA Wash Tubes	50 × 2
RNA Recovery Tubes	50 × 2

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SuperScript™ III First-Strand Synthesis SuperMix

Two boxes of SuperScript™ III First-Strand Synthesis SuperMix are provided. Sufficient reagents are provided to perform 100 reactions (50 reactions per box × 2). **Store all components at -20°C (non-frost-free).**

Component	Amount
SuperScript™ III/RNaseOUT™ Enzyme Mix	100 µl × 2
2X First-Strand Reaction Mix (contains 10 mM $MgCl_2$, and 1 mM each dNTP)	500 µl × 2
Annealing Buffer	50 µl × 2
Oligo$(dT)_{20}$ (50 µM)	50 µl × 2
Random hexamers (50 ng/µl)	50 µl × 2

StemPro® EZChek™ Human Tri-Lineage Primer Mix

One vial of StemPro® EZChek™ Human Tri-Lineage Primer Mix is provided, at a concentration of 10 µM in DNase/RNase-free water. Volume is provided to perform 100 20-µl PCR reactions. **Store at -20°C (non-frost-free).**

Component	Amount
StemPro® EZChek™ Human Tri-Lineage Primer Mix (10 µM)	100 µl

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Primer Sequences

The StemPro® EZChek™ Human Tri-Lineage Primer Mix contains the following PCR primers in a proprietary, optimized format:

Marker	Accession Number	Fragment Size	Primer	Sequence
GAPDH	NM_002046	983 bp	Forward	TGAAGGTCGGAGTCAACGGATTTGGT
			Reverse	CATGTGGGCCATGAGGTCCACCAC
Pou5f1/ Oct4	NM_002701	536 bp	Forward	GCAATTTGCCAAGCTCCTGAAGCAG
			Reverse	CATAGCCTGGGGTACCAAAATGGGG
AFP	NM_001134	400 bp	Forward	GAAATGACTCCAGTAAACCCTGGTG
			Reverse	AGACTCGTTTTGTCTTCTCTTCCCC
ACTC1	NM_005159	315 bp	Forward	CATCCTGACCCTGAAGTATCCCATC
			Reverse	CCCTCATAGATGGGGACATTGTGAG
SOX1	NM_005986	202 bp	Forward	GTGTCCAATTGTTGGCATCTAGGTC
			Reverse	CAAGGAAATAAGGTGGTTGGAGCAC

Accessory Products

Additional Products

The products listed in this section may be used with the StemPro® EZChek™ Human Tri-Lineage Multiplex PCR Kit. For more information, refer to our Web site (www.invitrogen.com) or call Technical Service (see page 20).

Item	Quantity	Catalog no.
AccuPrime™ *Pfx* SuperMix	200 Reactions	12344-040
RNase H	30 units	18021-014
RNase *AWAY*® Reagent	250 ml	10328-011
DNase I, Amplification Grade	100 units	18068-015
BG01V/hOG Cells (Variant hESC hOct4-GFP Reporter Cells)	~2 × 10^6 cells	R7799-105
StemPro® EZPassage™ Disposable Stem Cell Passaging Tool	10 tools	23181-010
StemPro® hESC SFM	1 kit	A10007-01
bFGF (full length) REC HU	100 µg	PHG0261
β-Mercaptoethanol	50 ml	21985-023
Collagenase Type IV	1 g	17104-019
Geltrex™	5 ml	12760-021
D-PBS (1X), liquid, without calcium, magnesium, or phenol red	500 ml 1,000 ml	14190-144 14190-136
TrypLE™ Select (1X), liquid	500 ml	12563-029
Trypan Blue Stain	100 ml	15250-061
DNase I, Amplification Grade	100 units	18068-015
BlueJuice™ Gel Loading Buffer	3 × 1 ml	10816-015
Qubit™ Fluorometer	1 fluorometer	Q32857
Qubit™ Quantitation Starter Kit	1 fluorometer + 4 assay kits	Q32860
Quant-iT™ RNA Assay Kit	1 kit	Q3310-40
TrackIt™ 100-bp DNA ladder	100 applications	10488058
PureLink™ Micro-to-Midi™ Total RNA Purification System	50 isolations	12183-018
SuperScript™ III First-Strand Synthesis SuperMix	50 reactions	18080-400

Introduction

Overview

Human embryonic stem cells (hESCs) and pluripotent human embryonal carcinoma stem cells (hECs) require continuous monitoring of their differentiation state and potential during early growth and maintenance (Junying Yu, 2006). This can be done using early differentiation markers.

The StemPro® EZChek™ Human Tri-Lineage Multiplex PCR Kit uses three early differentiation markers, one pluripotency marker, and a GAPDH control for characterizing hESCs and hEC cell populations under *in vitro* conditions. This kit allows you to rapidly and reliably monitor the differentiation state and potential of hESCs or hECs using a convenient reverse transcription-polymerase chain reaction (RT-PCR) assay.

Using the kit, you first isolate total RNA from cells, and then generate cDNA from the RNA using SuperScript™ III Reverse Transcriptase in a convenient supermix format. You then amplify the markers noted above in a highly sensitive, single-tube multiplex PCR reaction, using five different primer pairs. Finally, you run the results on an agarose gel to visualize the targets.

Kit Components

The StemPro® EZChek™ Human Tri-Lineage Multiplex PCR Kit contains the following components:

- **The PureLink™ Micro-to-Midi™ Total RNA Purification System** rapidly and reliably isolates high-quality total RNA from your hESCs or hECs.
- **SuperScript™ III First-Strand Synthesis SuperMix** provides high yields of first-strand cDNA
- **StemPro® EZChek™ Human Tri-Lineage Primer Mix** contains primers for detecting the following human genes in a multiplex PCR reaction:
    - Pou5f1/Oct4: marker for the pluripotent state of hESCs and hECs
    - AFP: endoderm lineage marker
    - ACTC1: mesoderm lineage marker
    - SOX1: ectoderm lineage marker
    - GAPDH: internal RNA standard for normalizing mRNA levels

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Introduction, Continued

Multiplex PCR

Multiplex polymerase chain reaction (PCR) is a variant of PCR in which two or more loci are simultaneously amplified in the same reaction. Since its first description in 1988 (Chamberlain *et al.*, 1988), this method has been successfully applied in many areas of DNA testing, including analyses of deletions, mutations and polymorphisms or quantitative assays and reverse transcription PCR (Henegariu *et al.*, 1997).

Markers Used

Gene expression studies of undifferentiated hES cells show that **Pou5f1** (previously known as **Oct4**) is closely associated with the pluripotent state in both mES and hESCs. It is essential for the development of the pluripotent inner cell mass (ICM) in human embryogenesis and is observed to be strongly down-regulated upon differentiation. At day 13 of hESC differentiation, expression of the pluripotent genes is greatly reduced (Bhattacharya *et al.*, 2005).

In 7-day differentiated embryoid bodies (EBs), expression of AFP is strongly up-regulated (Adewumi *et al.*, 2007). **AFP**, **ACTC1**, and **SOX1** markers can reliably detect the differentiation of hESCs into endoderm, mesoderm, and ectoderm lineages, respectively. These markers can be used for routine examination of differentiation in hESC cultures.

GAPDH has emerged as commonly used internal standard in ES cell-derived gene transcription studies for normalizing mRNA levels in quantitative analysis (Murphy & Polak, 2002).

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Recommended PCR Enzyme

AccuPrime™ *Pfx* SuperMix is recommended for use with this kit. It provides robust, highly specific amplification in demanding multiplex PCR applications. Ordering information is provided on page viii.

AccuPrime™ *Pfx* SuperMix includes recombinant DNA polymerase from *Thermococcus* species KOD, anti-KOD antibodies, thermostable AccuPrime™ proteins, $MgSO_4$, dNTPs, and stabilizers in a SuperMix formulation (Takagi *et al.*, 1997).

This highly processive enzyme is provided in an antibody-bound form that is inactive at ambient temperatures. The enzyme regains activity after the initial denaturation step at 94°C in PCR cycling, providing an automatic "hot start" that increases specificity, sensitivity, and yield, while allowing room temperature assembly (Sharkey *et al.*, 1994).

Thermostable AccuPrime™ proteins enhance specific primer-template hybridization during every cycle of PCR (Rapley, 1994). AccuPrime™ *Pfx* SuperMix is supplied at 1.1X concentration to allow approximately 10% of the final reaction volume to be used for the addition of primer and template solutions.

Advantages of the Kit

The StemPro® EZChek™ Human Tri-Lineage Multiplex PCR Kit provides the following advantages:

- Detects markers for all three lineages and the undifferentiated state in one PCR reaction using a convenient multiplex RT-PCR assay
- Faster and requires smaller sample volumes than traditional characterization methods, such as immunocytochemistry
- Enables monitoring of the differentiation potential of cultured hESCs and hECs after test cultures have been allowed to differentiate
- Contains reagents necessary to quickly isolate RNA and generate cDNA from hESCs and hECs, for use in multiplex PCR

Methods

Harvesting Cells

Introduction

This section provides instructions for harvesting hESCs and hECs. If using cells from a culture vessel with a different surface area, adjust volumes of reagents accordingly.

Amount of Cells Required

In general, we recommend using ≥1 × 10^6 cells with this kit. The kit was developed using cells grown in 6-cm^2 tissue-culture dishes and 6-well tissue-culture plates, though 12-well or 24-well plates may also yield sufficient numbers of cells. The columns provided with the PureLink™ Micro-to-Midi Total RNA Purification System can handle sample volumes up to 1 × 10^8 cells.

Materials Needed

You will need the following items in addition to the components provided in the kit:

- RNase-free tubes, 15 ml
- RNase-free pipette tips
- Tabletop centrifuge
- D-PBS (1X), liquid, without calcium, magnesium, or phenol red
- TrypLE™ Select (1X), liquid

Notes on Harvesting Cells

- Always wear disposable gloves while handling samples and reagents to prevent RNase contamination.
- Work quickly during sample harvesting, use RNase-free dissection tools and containers (scalpels, dishes, tubes etc.), and work on RNase-free work surfaces (use RNase *AWAY*® Reagent).
- Perform all steps on ice unless noted otherwise.
- If you will be purifying total RNA from fresh samples, keep samples on ice immediately after harvesting; quickly proceed to sample lysis and homogenizaton.
- To freeze samples, place them immediately after harvesting in liquid nitrogen or on dry ice. Keep frozen samples at -80°C or in liquid nitrogen until proceeding to sample lysis and homogenization.

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Harvesting Cells, Continued

Note about Feeder Cells

This kit has been tested using hESCs and hECs grown on **Murine embryonic fibroblast (MEF) feeder cells**. We have verified that the primers in this kit will not amplify sequences from these feeder cells.

If you are using other types of feeder cells, you may:

- Culture cells under feeder-free conditions before testing.
- Prepare a control containing only feeder cells to identify any sequences from these feeders that may be amplified by the primers in this kit.

Harvesting Cells

1. Pre-warm TrypLE™ Select to 37°C.
2. Remove media from tissue-culture dish, and rinse cells once with D-PBS (1X, without calcium, magnesium, or phenol red).
3. Treat cells with 5 ml of pre-warmed TrypLE™ Select and let stand for a few minutes.
4. Harvest cells and transfer to one or more 15-ml centrifuge tubes on ice.
5. Take 100 µl of cells and perform a trypan blue viable cell count.
6. Spin tube(s) containing cells in a tabletop centrifuge for 3–5 minutes at 100 × *g* to pellet the cells. Discard the supernatant.
7. If proceeding directly to RNA isolation, place samples on ice; alternatively, freeze samples on dry ice or liquid nitrogen and store in –80°C freezer.

Isolating Total RNA

Introduction

This section provides instructions for preparing total RNA from harvested hESCs and hECs. Review the information in this section before starting. Guidelines are provided for handling RNA, handling system reagents, and lysis and homogenization.

Materials Needed

You will need the following items in addition to the components provided in the kit:

- 2-mercaptoethanol
- 96–100% ethanol
- 70% ethanol (in RNase-free water)
- Microcentrifuge capable of centrifuging 12,000 × *g*
- 1.5-ml RNase-free microcentrifuge tubes
- RNase-free pipette tips

Guidelines for Handling RNA

Follow the guidelines below to prevent RNase contamination and maximize the RNA yield:

- Use disposable, individually wrapped, sterile plasticware.
- Use only sterile, disposable RNase-free pipette tips and microcentrifuge tubes.
- Wear disposable gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin. Change gloves frequently, particularly as the protocol progresses from crude extracts to more purified material (*e.g.*, from Wash Buffer I to Wash Buffer II).
- Always use proper microbiological aseptic techniques when working with RNA.
- Use RNase *AWAY*® Reagent (for catalog number, see page viii) to remove RNase contamination from work surfaces and non-disposable items such as centrifuges and pipettes used during purification.

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Isolating Total RNA, Continued

Some of the PureLink™ Micro-to-Midi™ Total RNA Purification System buffers contain hazardous chemicals.

- Both the RNA Lysis Solution and Wash Buffer I contain guanidine isothiocyanate. This chemical is harmful if it comes in contact with the skin or is inhaled or swallowed. Always wear a laboratory coat, disposable gloves, and goggles when handling solutions containing this chemical.
- Do not add bleach or acidic solutions directly to solutions containing guanidine isothiocyanate or sample preparation waste. Guanidine isothiocyanate forms reactive compounds and toxic gases when mixed with bleach or acids.
- Solutions containing ethanol are considered flammable. Use appropriate precautions when using this chemical.
- Always wear a laboratory coat, disposable gloves, and eye protection when handling buffers. Dispose of the buffers in appropriate waste containers.

Preparing Wash Buffer II with Ethanol

Before using the Wash Buffer II for the first time, add 60 ml of 96–100% ethanol directly to the bottle. Check the box on the Wash Buffer II label to indicate that ethanol was added.

Preparing RNA Lysis Solution with 2-Mercapto-ethanol

Prepare the amount of RNA Lysis Solution needed for the purification procedure fresh for each use by adding 1% (v/v) 2-mercaptoethanol. Add 10 µl of 2-mercaptoethanol to each 1 ml of RNA Lysis Solution.

Continued on next page

Isolating Total RNA, Continued

Cell Lysis

After harvesting the cells as described on page 5, proceed with the steps below.

Note: For frozen samples, thaw before proceeding**.**

1. To the tube containing the cell pellet, add 0.5 ml of RNA Lysis Solution (prepared with 2-mercaptoethanol) per 1–5 × 10^6 cells. Note that one 6-cm^2 tissue-culture dish typically contains 2–5 × 10^6 cells.
2. Pipet cells up and down or vortex until cells are disrupted.
3. Transfer 0.5-ml aliquots of lysed cells to individual 1.5-ml RNAse-free microcentrifuge tubes.
4. Centrifuge tubes for 2 minutes at 12,000 × *g*, room temperature.
5. Add 0.5 ml 70% EtOH to each tube.
6. Pipet suspension up and down 5–10 times.

Binding, Washing, and Elution

1. Transfer a 600-µl aliquot of cell lysis solution to an RNA Spin Cartridge, inserted in a collection tube.
2. Centrifuge for 15–30 seconds at 12,000 × *g*, room temperature. Discard flow-through.
3. Repeat Steps 1–2 until entire sample has been processed.
4. Add 700 µl of Wash Buffer I to the cartridge.
5. Centrifuge for 15–30 seconds at 12,000 × *g*, room temperature.
6. Discard flow-through and tube. Place cartridge into clean 2-ml RNA Wash Tube.
7. Add 500 µl Wash Buffer II (prepared with ethanol) to cartridge and centrifuge for 15–30 seconds at 12,000 × *g*, room temperature.
8. Discard flow-through. Centrifuge for 1 minute to dry cartridge.
9. Place cartridge into RNA Recovery Tube. Add 40 µl of RNAse-free water to cartridge.
10. Let stand for 1 minute, then centrifuge for 2 minutes at 12,000 × *g*, room temperature. The eluate contains the purified total RNA.

Proceed to **Analyzing RNA Yield and Quality**, next page.

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Isolating Total RNA, Continued

Determining RNA Yield

Quant-iT™ Kits

Quant-iT™ RNA assays from Invitrogen provide a rapid, sensitive, and specific fluorescent method for RNA quantitation. Each kit contains a state-of-the-art quantitation reagent and a pre-made buffer to allow quantitation using standard fluorescent microplate readers/fluorometers or the Qubit™ Quantitation Fluorometer. See page viii for ordering information. Visit www.invitrogen.com/naprep for more information.

UV Absorbance

1. Dilute an aliquot of the purified total RNA in RNase-free water (*i.e.,* elution buffer).
2. Blank the UV/visible spectrophotometer using RNase-free water, then scan the sample at 260 nm.
3. Calculate the yield of RNA using the formula:

   Total RNA yield (ng/µl) = A_{260} × 40 (constant for RNA in ng/µl) × dilution factor

   For example, if the A_{260} is 0.2 and the total RNA has been diluted 1:50, then 0.2 × 40 ng/µl × 50 = 400 ng/µl.

Expected Yield

Typical yield for hESCs and hECs harvested as described previously is 300–800 ng/µl. If the concentration is below 200 ng/µl, we recommend resuspending the RNA pellet in a lower volume (*e.g.,* 5–20 µl) before proceeding to cDNA synthesis.

Determining RNA Quality

The quality of the purified total RNA can be analyzed using a bioanalyzer such as the Agilent 2100 bioanalyzer with an RNA LabChip®.

Alternatively, total RNA quality can be analyzed by agarose gel electrophoresis. RNA isolated using the PureLink™ kit typically has a 28S-to-18S band ratio of >1.5. RNA is judged to be intact if discreet 28S and 18S ribosomal RNA bands are observed.

cDNA Synthesis

Introduction

This section provides instructions for synthesizing cDNA from total RNA using the components of the SuperScript™ III First-Strand Synthesis SuperMix kit.

Materials Needed

You will need the following items in addition to the components provided in the kit:

- Ice bucket
- RNase H
- Thermocycler
- RNase-free microcentrifuge tubes
- RNase-free pipette tips
- **Optional:** DNase I, Amplification Grade

DNase I Treatment

DNase I treatment prior to cDNA synthesis is typically not required if you isolated RNA using the PureLink™ Micro-to-Midi Total RNA Purification System included with this kit. If you are using RNA from other sources, you may need to perform a DNase I treatment to digest genomic DNA. Refer to the protocol **DNase I Treatment of RNA** in the Appendix (page 20).

Guidelines for Handling RNA

Follow the standard guidelines for handling RNA as described on page 6 to prevent RNase contamination.

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cDNA Synthesis, Continued

cDNA Synthesis

Note that the following protocol uses the random hexamers provided with the SuperScript™ III First-Strand Synthesis SuperMix kit:

1. Mix and briefly centrifuge each component before use. Preheat the thermal cycler to 65°C.
2. Combine the following in a 0.2-ml PCR tube on ice. Use the yield calculations on page 9 to determine the volume containing 1 µg of total RNA.

Component	
1 µg total RNA	*n* µl
50 ng/µl random hexamers	1 µl
Annealing Buffer	1 µl
RNase/DNase-free water	to 8 µl

3. Incubate in a thermal cycler at 65°C for 5 minutes, and then immediately place on ice for at least 1 minute. Collect the contents of the tube by brief centrifugation.
4. Add the following to the tube on ice:

2X First-Strand Reaction Mix	10 µl
SuperScript™ III/RNaseOUT™ Enzyme Mix	2 µl

5. Vortex the sample briefly to mix, and collect by brief centrifugation.
6. Incubate 10 minutes at 25°C.
7. Incubate 50 minutes at 42°C.
8. Terminate the reaction at 85°C for 5 minutes. Chill on ice.
9. Add 1 µl RNAse H and incubate at 37°C for 20 minutes.

Proceed to **Multiplex PCR Amplification**, next page.

Multiplex PCR Amplification

Introduction

This section provides instructions for amplifying cDNA using AccuPrime™ *Pfx* DNA Polymerase (purchased separately) in a multiplex PCR reaction.

Materials Needed

You will need the following items:

- Ice bucket
- Thermocycler
- PCR enzyme (AccuPrime™ *Pfx* SuperMix recommended)
- PCR grade microcentrifuge tubes
- PCR grade pipette tips

Note on PCR Enzyme

AccuPrime™ *Pfx* SuperMix (described on page 3) has been tested and is recommended for use with the primer mix provided in this kit. See page viii for ordering information.

A protocol using this supermix is provided on the following page. Other polymerases may achieve comparable results.

Guidelines for Performing PCR

- PCR is a powerful technique capable of amplifying trace amounts of DNA; take all appropriate precautions to avoid cross-contamination.
- For multiple reactions, you can prepare a master mix of the DNA polymerase and the component(s) common to all reactions.
- All steps are done on ice unless noted otherwise. For all incubations, thermocyclers were pre-heated in advance. All reagents are pre-chilled/frozen and thawed immediately prior to use.

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Multiplex PCR Amplification, Continued

Multiplex PCR Amplification

The following protocol uses AccuPrime™ *Pfx* SuperMix. If you are using a different DNA polymerase, follow the protocol provided with that enzyme, scaling the volume of StemPro® EZChek™ Human Tri-Lineage Primer Mix accordingly.

No-template controls (NTCs): We recommend setting up no-template control (NTC) reactions to check for spurious amplification products.

1. For a single reaction, combine the following components in a PCR tube or well of 96-well PCR plate.

Component	Single rxn
AccuPrime™ *Pfx* SuperMix	18 µl
StemPro® EZChek™ Human Tri-Lineage Primer Mix	1 µl
Template cDNA (from page 11)	1 µl

2. Prepare NTC reactions using the mix above but replacing the template cDNA with distilled water.
3. Transfer the reactions to a thermal cycler and run the following cycling program:

   a. Initial denaturation at 95 ºC for 2 minutes
   b. 30 cycles of:
      95ºC, 30 seconds
      60ºC, 30 seconds
      68ºC, 1 minute
   c. Final extension at 68ºC for 5 minutes

4. Maintain reaction at 4°C after cycling. Samples can be stored at -20°C.

Analyzing the Results

Introduction

Following amplification, run the PCR products on a gel to identify the bands.

Materials Needed

You will need the following items in addition to the components provided in the kit:

- 2% agarose gel
- Molecular weight marker with bands between 100 bp and 1000 bp (*e.g.*, the TrackIt™ 100-bp DNA Ladder)

Gel Analysis

1. Load the 20-µl PCR reactions in separate wells of a 2% agarose gel.
2. In an adjacent lane, load 20 µl of a molecular weight marker to estimate the size of the PCR products.
3. Load 20 µl of water into any empty wells.
4. Run the gel for 30 minutes.
5. Visualize bands on a UV transilluminator. Use a gel imaging system to determine the intensities of the bands.

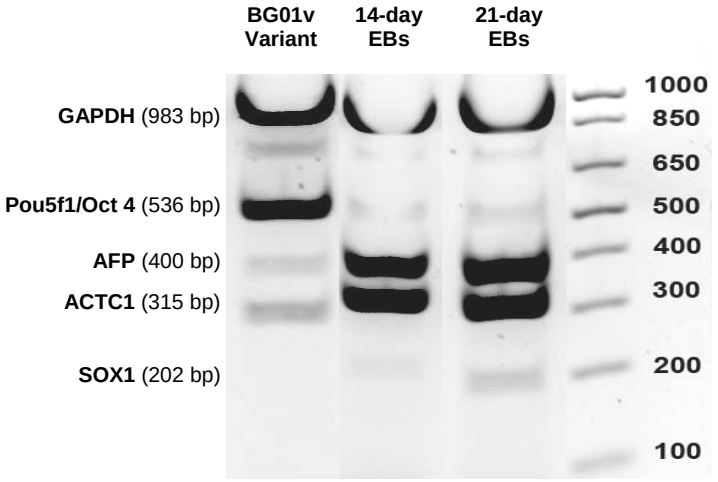
Note on Band Intensities

When studying differentiation markers on the gel, note that band presence or absence is more important than band intensity. SOX1 in particular may appear quite faint, due to the nature of neural stem cell differentiation.

Example Results

Example Results — BG01v stem cells and 14-day and 21-day EBs

In the example below, BG01v stem cells and 14-day and 21-day EBs were analyzed using the StemPro® EZChek™ Human Tri-Lineage Multiplex PCR Kit. The gel shows that Pou5f1/Oct4 expression was down-regulated in the BG01v EBs versus the stem cells, while the differentiation markers AFP and ACTC1 were clearly induced in the EBs. SOX1 was also induced, most visibly in the 21-day EBs.



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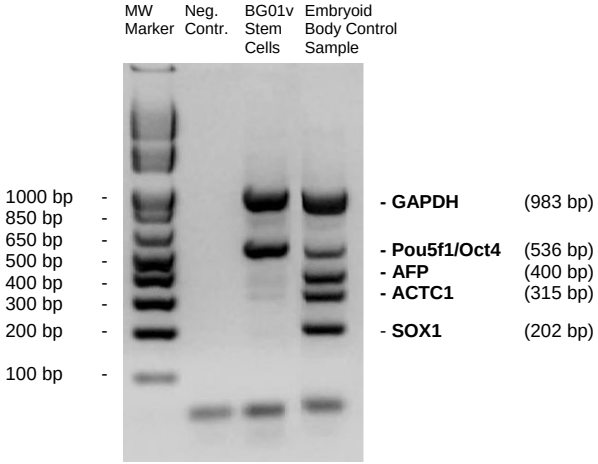
Example Results, Continued

Example Results — EB Control Sample

In the example below, BG01v stem cells and an EB control sample were analyzed using the StemPro® EZChek™ Human Tri-Lineage Multiplex PCR Kit. The gel shows that Pou5f1/Oct4 expression was down-regulated in the BG01v EBs versus the stem cells. In addition, the differentiation markers AFP and ACTC1 were induced in the EB control sample. Since the intensity of the SOX1 signal was relatively low, DNA from SOX1 BAC was spiked into the cDNA from the 21-day EBs to generate a control sample with equal band intensities for all markers.



Troubleshooting

Problem	Cause	Solution
Clogged RNA Spin Cartridge	Incomplete homogenization or dispersal of precipitate after ethanol addition	• Follow protocol guidelines for each sample type and amount. • Clear homogenate and remove any particulate or viscous material by centrifugation and use only the supernatant for subsequent loading on to the RNA Spin Cartridge. • Completely disperse any precipitate that forms after adding ethanol to the homogenate.
Low RNA yield	Incomplete lysis and homogenization	• Ensure that 10 µl of 2-mercaptoethanol was added per 1 ml of RNA Lysis Solution. • Perform all steps at room temperature unless directed otherwise. • Decrease the amount of starting material used.
	Poor quality of starting material	Be sure to use fresh sample and process immediately after collection or freeze the sample at -80ºC or in liquid nitrogen immediately after harvesting.
	Ethanol not added to Wash Buffer II	Be sure that ethanol was added to Wash Buffer II as directed on page 7.
	Incorrect elution conditions	• Add RNase-free water and perform incubation for 1 minute before centrifugation. • To recover more RNA, perform a second elution step.

Continued on next page

Troubleshooting, Continued

Problem	Cause	Solution
RNA degraded	RNA contaminated with RNase	• Use RNase-free pipet tips with aerosol barriers. • Change gloves frequently. • Swipe automatic pipettes with RNase AWAY™ solution after washing RNA Spin Cartridge with Wash Buffer I.
	Improper handling of sample from harvest until lysis	• If not processed immediately, quick-freeze tissue immediately after harvesting and store at –80ºC or in liquid nitrogen. • Frozen samples must remain frozen until RNA Lysis Solution was added. • Perform the lysis quickly after adding RNA Lysis Solution
Inhibition of downstream enzymatic reactions	Presence of ethanol in purified RNA	Traces of ethanol from the Wash Buffer II can inhibit downstream enzymatic reactions. Discard Wash Buffer II flow through. Place the RNA Spin Cartridge into the Wash Tube and centrifuge the spin cartridge at maximum speed for 2–3 minutes to completely dry the cartridge.
	Presence of salt in purified RNA	Use the correct order of Wash Buffers for washing. Always wash the cartridge with Wash Buffer I followed by washing with Wash Buffer II.
Bands in gel appear weak or faint	Suboptimal DNA polymerase used in PCR	We recommend using AccuPrime™ *Pfx* SuperMix as described on page 13. Note that the DNA polymerase must be capable of amplifying five distinct targets in a single reaction.
	Procedural error in first-strand cDNA synthesis	Repeat the procedure, being careful to follow each step. Be careful to include the Annealing Buffer when adding primers and template for optimal yield.
	RNase contamination	Maintain aseptic conditions to prevent RNase contamination. RNaseOUT™ is included in the Enzyme Mix to inhibit RNases.

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Troubleshooting, Continued

Problem	Cause	Solution
Unexpected bands appear in lanes	PCR primers are amplifying sequences from feeder cells	This kit has been tested using hESCs and hECs grown on Murine embryonic fibroblast (MEF) feeder cells. We have verified that the primers in this kit will not amplify sequences from these feeder cells. If you are using other feeder cells, prepare a feeder cell-only reaction to check for amplified products. If products are evident, culture hESCs and hECs under feeder-free conditions before testing.
	Contamination by genomic DNA	Prior to cDNA synthesis, treat RNA preparation with DNase I, Amplification Grade (Cat. no. 18068-015), as described on page 20.

Appendix

DNase I Treatment of RNA

Introduction

This section provides instructions for treating total RNA with DNase I to digest genomic DNA. You do not have to perform a DNase I treatment before starting the cDNA synthesis if you isolated RNA using the protocol described in **Isolating Total RNA** (page 6). If you are using RNA from other sources, you may need to perform a DNase I treatment to digest genomic DNA.

DNase I Treatment

1. Set up RNase-free, 0.5-ml microcentrifuge tubes on ice.
2. Add to each tube the following solutions:

Component	Volume
RNA sample, 1 µg	x µl
10X DNase I Reaction Buffer	1 µl
DNase I amplification grade, 1 U/µl	1 µl
DEPC-treated water	to 10 µl

Note: To work with larger amounts of RNA, scale up the reaction (including volume) linearly.

3. Incubate tubes for 15 minutes at room temperature.
4. Inactivate the DNase I by the addition of 1 µl of 25 mM EDTA solution to the reaction mixture. Heat for 10 minutes at 65°C. The RNA sample is ready to use in reverse transcription, prior to amplification.
5. Store RNA samples at -70°C.

Product Qualification

Product Qualification

The **PureLink™ Micro-to-Midi™ Total RNA Purification System** and **SuperScript™ III First-Strand Synthesis SuperMix** are qualified as described in the Certificate of Analysis (CofA) for each product, available on our website by product lot number at www.invitrogen.com/cofa. Note that the lot number is printed on the box for each product.

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