Human Pluripotent Stem Cell Assessment Primer Pair Panel

Catalog Number SC012

Reagents for the identification of human stem cell differentiation states.

This package insert must be read in its entirety before using this product.
For laboratory research use only. Not for diagnostic use.
The safety and efficacy of this product in diagnostic or
other clinical uses has not been established.

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614 McKinley Place NE, Minneapolis, MN 55413, USA
TEL: (800) 343-7475 (612) 379-2956 FAX: (612) 656-4400
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INTRODUCTION

Embryonic stem (ES) cells, derived from the inner cell mass of pre-implantation embryos, have been recognized as the most pluripotent stem cell population (1, 2). It has been demonstrated that cells are able to be reprogrammed to an ES cell-like state (3). These human ES cells and human induced pluripotent stem cells (iPS) can be maintained and propagated for extended periods in media containing fibroblast growth factor basic (FGF basic) (4). Differentiation of ES/iPS cells to a lineage-committed fate is typically monitored by characterization of its phenotypic properties. More recently, molecular markers for ES/iPS cell differentiation have been identified by a variety of techniques including comparison with databases, reverse transcription-polymerase chain reaction (RT-PCR*), focused cDNA microarrays, and immunocytochemistry. The identification of a common set of expressed genes contributing to the core properties of ES/iPS cells, or their "stemness" phenotype has been established (4-12).

MATERIALS PROVIDED

The Human Pluripotent Stem Cell Assessment Primer Pair Panel profiles the mRNA transcripts of fourteen genes that are frequently used as markers for molecular characterization of undifferentiated and lineage-committed human ES and iPS cells (Table 1). A primer pair for human GAPDH is included and can be used as a control for successful cDNA synthesis. A positive control is also included. See Table 2 in the appendix.

Table 1: *Expected gene expression patterns detected by this panel.*

UNDIFFERENTIATED ES/IPS CELLS	ECTODERMAL LINEAGE	ENDODERMAL LINEAGE	MESODERMAL LINEAGE	GERM CELLS
DPPA5/ESG1	Nestin	AFP	Brachyury	Stella
Nanog	Otx2	GATA-4	——	——
Oct-3/4	TP63	PDX-1	——	——
SOX2	SOX2	SOX17	——	——
——	——	HNF-3β	——	——

Primer Pairs - Each vial contains 375 pmoles of lyophilized primer. See Table 2 in the appendix for details about the primer pairs provided in this kit.

Positive Control 57 - One vial containing 1500 ng of lyophilzed synthetic double-stranded DNA. The Positive Control is not intended for quantitative purposes.

**PCR is covered by U.S. Patent numbers 4683195 and 4683202 assigned to Hoffmann-La Roche.*

OTHER SUPPLIES REQUIRED

Reagents

- RNase-free DNase
- Random Primers (R&D Systems, Catalog # RDPC2) or Oligo $(dT)_{12-18}$ primers (R&D Systems, Catalog # RDPC1)
- RNase H^- Reverse Transcriptase
- Taq DNA Polymerase
- Agarose
- Reverse Transcription Buffer
- 10X Taq Buffer
- 2 mM dNTPs
- 10 mM dNTPs
- 0.1 M DTT
- Autoclaved deionized water (dH_2O)
- Nuclease-free dH_2O
- Gel loading dye
- DNA markers
- Mineral oil
- 0.1X TE
- 1X TAE

Equipment

- Thermal cycler (Stratagene Robocycler or equivalent)
- Hot block or water bath at 70 °C
- Hot block or water bath at 42 °C
- Thin walled PCR tubes
- 1.5 mL sterile tubes
- Power supply

Disposables

- Pipettes and aerosol barrier pipette tips
- Gloves

LIMITATIONS OF THE PROCEDURE

- FOR LABORATORY RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The safety and efficacy of this product in diagnostic or other clinical uses has not been established.
- The kit should not be used beyond the expiration date on the kit label.
- The quality of the cells and any variations in the procedure can cause variation in the results.

STORAGE CONDITIONS

The reconstituted primer pairs are stable for up to one year at ≤ -20 °C* in a manual defrost freezer. Aliquot in single use portions. Avoid repeated freeze-thaw cycles.

*Provided this is within the expiration date of the kit.

SPECIFICITY

Twelve of the fourteen pluripotent stem cell primer pairs are specific for human cDNA. All primer pairs were also tested with mouse and rat cDNA. The SOX17 and Brachyury primer pairs are not optimized for use with mouse or rat cDNA since they very weakly amplify mouse and rat cDNA. All pluripotent stem cell primer pairs were tested with mouse embryonic fibroblast (MEF) cDNA and did not amplify a product.

RECOMMENDED PROTOCOLS

I. DNase Treatment

Treatment of total RNA with RNase-free DNase is required to remove contaminating genomic DNA. Follow the procedure according to the manufacturer's instructions. If DNase treatment of RNA is not done, false positives may result.

II. Reverse Transcription Reaction

1. Thaw all reagents completely on ice. All reactions should be assembled on ice.
2. Pipette the following into a nuclease-free tube:
   - 1-5 µg of DNase treated total RNA (up to 11 µL)
   - 1 µL of random primers (300 ng/µL) or Oligo $(dT)_{12-18}$ primers (0.5 µg/µL)
   - X µL of nuclease-free dH_2O for a final volume of 12 µL
3. Mix and incubate at 70 °C for 10 minutes. Immediately place the tube on ice.
4. Briefly centrifuge the tube, and add the following to each tube:
   - 4 µL of 5X Reverse Transcription Buffer
   - 2 µL of 0.1 M DTT
   - 1 µL of 10 mM dNTPs
5. Mix and incubate at room temperature (18-24 °C) for 10 minutes.
6. Incubate at 42 °C for 2 minutes.
7. Add 1 µL of RNase H^- Reverse Transcriptase (200 units/µL). Mix by pipetting.
8. Incubate at 42 °C for 50 minutes.
9. Incubate at 70 °C for 15 minutes.
10. Dilute reactions 5-fold by adding 80 µL of nuclease-free dH_2O.
11. Assay immediately, or store the cDNA samples at ≤ -20 °C in a manual defrost freezer.

III. PCR Reaction

1. Resuspend each Primer Pair in 50 µL of autoclaved deionized water or 0.1X TE Buffer (1 mM Tris HCl, 0.1 mM EDTA, pH 8.0 at 25 °C) for a final concentration of 7.5 pmoles/µL for each primer. Do not resuspend the Positive Control at this time.

2. Determine the number of PCR reactions (see Technical Hints for setting up a PCR master mix). Multiply the volumes listed below for each reagent by the number of reactions. Prepare a separate master mix for each primer pair.
   - 36.5 µL of autoclaved deionized water
   - 5 µL of 10X Taq Buffer (with 15 mM $MgCl_2$)
   - 5 µL of 10X dNTP mix (10X = 2 mM each dNTP
   - 2 µL of Primers (7.5 pmoles/µL each primer)
   - 0.5 µL of Taq DNA Polymerase (5 units/µL)

3. Prepare the Negative Control reaction tube:
   a. Pipette 1 µL of autoclaved deionized water into a pre-labeled negative control tube.
   b. Add 49 µL of the master mix prepared in step 2.
   c. Briefly spin the tube, and add 30 µL of mineral oil to prevent evaporation.
   d. Close the reaction tube and place on ice.

4. Prepare the cDNA sample reaction tube(s):
   a. Pipette 1 µL of cDNA sample into a pre-labeled PCR reaction tube.
   b. Add 49 µL of the master mix prepared in step 2.
   c. Briefly spin the tube, and add 30 µL of mineral oil to prevent evaporation.
   d. Close the reaction tube and place on ice.

5. Resuspend Positive Control 57 in 150 µL of autoclaved deionized water or 0.1X TE Buffer. Centrifuge the Positive Control tube briefly. This should be done in a separate location from where PCR reactions are set up. Use different pipettes than those used for PCR set up.
   a. Pipette 1 µL of Positive Control into a pre-labeled positive control reaction tube.
   b. Add 49 µL of the master mix prepared in step 2.
   c. Briefly spin the tube, and add 30 µL of mineral oil to prevent evaporation.
   d. Close the reaction tube and place on ice.

6. Place all tubes in a thermal cycler, and perform the following program:
   - 94 °C for 4 minutes
   - 94 °C for 45 seconds
   - 55 °C for 45 seconds
   - 72 °C for 45 seconds

   } 30-35 cycles

   - 72 °C for 10 minutes

TECHNICAL HINTS

- Thaw all reagents completely on ice before use.
- Use either Random Primers or Oligo (dT) for Reverse Transcription.
- The recommended annealing temperature is 55 °C.
- To minimize the risk of amplicon contamination of the Primer Pairs and other PCR reagents, the following precautions are recommended:
  - PCR reactions should be set up in an area separate from where PCR products are analyzed.
  - Pipettes and tube racks should be specifically designated for PCR.
  - Use aerosol barrier pipette tips.
- Follow the steps below to determine the number of PCR reactions and to calculate the amount of PCR master mix necessary.
  a. Determine the number of cDNA samples that will be analyzed. Include a positive and negative control. Add 2 to the number of cDNA samples to account for the two controls.
  b. Multiply the number of samples by a factor of 1.1 to account for pipetting volume. This is the number of reactions for which the master mix should be made.

  For example, the master mix for 3 cDNA samples should be:
  - 3 cDNA samples will be analyzed.
  - Plus 2 for positive and negative controls = 5
  - 5 x 1.1 for pipetting volume = 5.5
- Primer Pairs are not validated for use in kinetic RT-PCR.

ANALYSIS OF RESULTS

The PCR products can be analyzed by 1.5-2.0% agarose gel electrophoresis. For predicted sizes of PCR products, refer to Table 2.

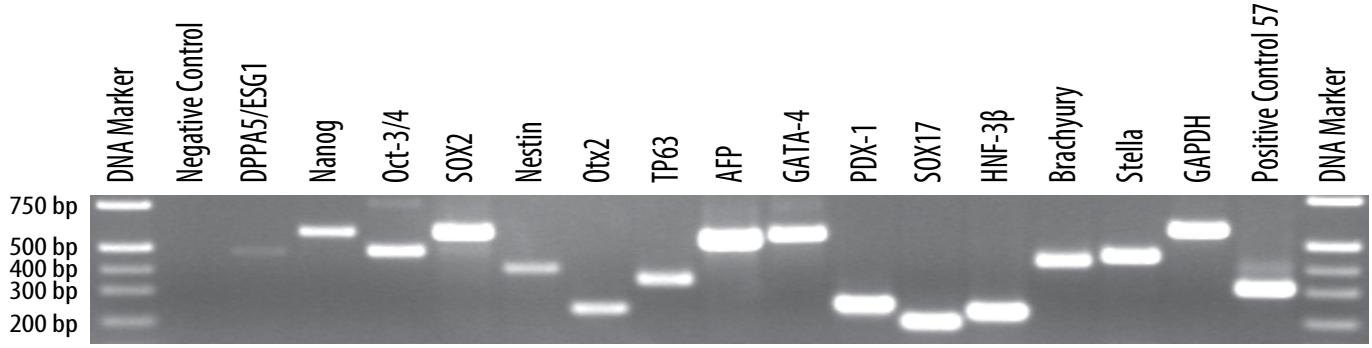

Figure 1: *PCR products derived from various tissue samples were separated by agarose gel electrophoresis.*

APPENDIX OF PCR PRODUCTS

Table 2: *Predicted sizes of PCR products provided in the Human Pluripotent Stem Cell Assessment Primer Pair Panel.*

GENE	PRIMER PAIR CATALOG #	GENBANK ACCESSION #	cDNA PRODUCT SIZE (bp)	GENOMIC PRODUCT SIZE (BP)	COMMENTS
AFP	RDP-324	NM_001134	537	3804	——
Brachyury	RDP-331	NM_003181	430	3387	——
DPPA5/ESG1	RDP-319	NM_001025290	484	1039	——
GAPDH	RDP-39	M33197	576	861	576 bp product also seen from pseudogenes.*
GATA-4	RDP-325	NM_002052	569	9413	——
HNF-3β	RDP-328	NM_021784 (variation 1) NM_153675 (variation 2)	230	230	Amplifies both variants to give the same size product.
Nanog	RDP-320	NM_024865	596	1974	596 bp product also seen from pseudogenes.*
Nestin	RDP-148	NM_006617	406	2341	——
Oct-3/4	RDP-321	NM_002701 (variation 1) NM_203289 (variation 2)	486	1031	486 bp product also seen from pseudogene. Amplifies both splice variants to give the same size product.*
Otx2	RDP-330	NM_021728 (variation 1) NM_172337 (variation 2)	235	2067	Amplifies both splice variants to give the same size product.*
PDX-1	RDP-326	NM_000209	262	3972	——
SOX2	RDP-323	NM_003106	591	591	——
SOX17	RDP-327	NM_022454	194	807	——
Stella	RDP-332	NM_199286	445	5471	445 bp product also seen from pseudogenes.*
TP63	RDP-329	NM_003722	358	2636	——
Positive Control 57	——	——	320	——	Synthetic double-stranded DNA.

bp = base pairs

**Pseudogenes will not affect performance in properly DNase-treated samples.*

TROUBLESHOOTING GUIDE

PROBLEM	SUGGESTIONS
No PCR products obtained.	Operator error may have occurred during reaction assembly. Run positive control reaction.
	Unsuccessful cDNA synthesis. Use the GAPDH primer pair as a control to show that the RT reaction was successful.
	RNA may be degraded. Check the integrity of the RNA by gel electrophoresis. A good quality RNA preparation should show the 28S and 18S ribosomal RNAs in an approximate 2:1 ratio. If RNA appears degraded, repeat the RNA isolation.
	RNA secondary structure may be inhibiting cDNA synthesis. This can often be overcome by using random primers.
	The target RNA concentration may be too low. Increase the amount of cDNA template used in the PCR reaction or the number of cycles used to amplify the cDNA.
Smearing or bands of unpredicted size.	Non-specific priming may have occurred. Use less cDNA or reduce the number of cycles.

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