Human ESC Germ Layer PCR Kit

Cat. No. SCR063

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Not for use in diagnostic procedures.

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Introduction

Culturing human embryonic stem cells requires specialized training and meticulous and diligent care. Even in the most optimal in vitro conditions, some degree of spontaneous differentiation is evident in the human ES colonies. It has become a routine and essential part of culturing human ES cells to visually inspect and remove differentiated cell colonies to ensure the overall health and undifferentiated status of the ES cell population. Consequences for neglect can be dire and include decreased cell proliferation, low cell viability upon freeze-thawing and also decreased capacity of cells to differentiate into the three embryonic germ layers (endoderm, mesoderm and ectoderm). Although important, the use of visual inspection alone can be subjective and qualitative.

Millipore's Human ESC Germ Layer PCR Kit enables researchers to quantitatively monitor the health of the starting undifferentiated human ESC population and to analyze the capacity of human ESC to differentiate into cell derivatives of the three germ layers. Provided in the kit are optimized and validated primer sets for two pluripotent markers (Nanog and Oct4), an endoderm marker (AFP), a mesoderm marker (α-MHC), an ectoderm marker (Pax6) and a constitutive marker (GAPDH) along with control cDNAs from undifferentiated human ESC and from human ESC that have been differentiated as embryoid bodies (EB). Nanog and Oct4 are essential transcriptional factors that help maintain the pluripotency of ESCs (2). The down-regulation of Nanog and Oct4 expressions are closely associated with progression of differentiation. Expressions of AFP, cardiac muscle specific myosin heavy chain 6 (α-MHC), and the neuronal marker, Pax6, indicate the presence of cells that have differentiated into endodermal, mesodermal and ectodermal lineages, respectively. AFP and Pax6 transcripts can be detected in differentiated embryoid bodies (EB) by RT-PCR by 7 days of differentiation (3, 4). The two cDNA controls are provided as useful bench-marks that can be used to measure the relative health and undifferentiated status of human ESC (ES cell cDNA control) and their capacity to give rise to cell derivatives of the three embryonic germ layers (EB cDNA control).

PCR conditions have been optimized and are provided. Millipore's Human ESC Germ Layer PCR kit is compatible for use with cDNAs that have been generated from commercially available kits for RNA isolation and reverse transcription. Control cDNAs from undifferentiated human ESC and differentiated EBs were generated from reverse transcription of high-quality total RNA using oligo-dT as the primer. DNA primers have been validated to be specific to human ES cells and do not recognize and amplify nucleic acids from murine sources. Because the primers do not recognize murine nucleic acids, the kit can be used on cDNAs generated from human ESC that have been cultured on murine embryonic fibroblasts (MEF).

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Kit Components

1. GAPDH primer set: (Part No. CS201372) 10 µM mixed primers, 50 µL, sufficient for 100 reactions.
2. Nanog primer set: (Part No CS201373) 10 µM mixed primers, 50 µL, sufficient for 100 reactions.
3. Oct4 primer set: (Part No CS201374) 10 µM mixed primers, 50 µL, sufficient for 100 reactions.
4. AFP primer set: (Part No CS201375) 10 µM mixed primers, 50 µL, sufficient for 100 reactions.
5. α-MHC primer set: (Part No CS201376) 10 µM mixed primers, 50 µL, sufficient for 100 reactions.
6. PAX-6 primer set: (Part No CS201377) 10 µM mixed primers, 50 µL, sufficient for 100 reactions.
7. Embryonic Stem Cell (ES) cDNA Control: (Part No CS201378) From total RNA at 10 ng/µL, 90 µL
8. Embryoid Body (EB) cDNA Control: (Part No CS201379) From total RNA at 10 ng/µL, 90 µL
9. Sterile distilled water : (Part No 90442) 1.3 mL
10. 5x loading dye: (Part No CS201380) 500 µL

Primer Sequences

Marker	Accession Number	Primer Sequence	Product Size
GAPDH	NM_002046.3	Forward 5'- AGC CAC ATC GCT CAG ACA CC -3'	302 bp
		Reverse 5'- GTA CTC AGC GCC AGC ATC G -3'	
NANOG	NM_024865.1	Forward 5' - GCT TGC CTT GCT TTG AAG CA -3'	256 bp
		Reverse 5' - TTC TTG ACC GGG ACC TTG TC -3'	
OCT4	NM_002701.4	Forward 5' - GAG CAA AAC CCG GAG GAG T -3'	310 bp
		Reverse 5' - TTC TCT TTC GGG CCT GCA C -3'	
AFP	NM_001134.1	Forward 5' - AGAACCTGTCACAAGCTGTG -3'	676 bp
		Reverse 5' - GAC AGC AAG CTG AGG ATG TC -3'	
α-MHC	NM_002471.2	Forward 5' - GTC ATT GCT GAA ACC GAG AAT G -3'	413 bp
		Reverse 5' - GCA AAG TAC TGG ATG ACA CGC T -3'	
PAX-6	NM_001604.3	Forward 5' - AAC AGA CAC AGC CCT CAC AAA CA -3'	275 bp
		Reverse 5' - CGG GAA CTT GAA CTG GAA CTG AC -3'	

Materials Required but Not Supplied

1. RNA isolation kit
2. Reverse transcriptase
3. Hot-start DNA polymerase.
4. Thin-wall PCR tubes
5. Agarose gel and electrophoresis apparatus and reagents

Precautions

Handle all samples and reagents with clean disposable gloves and use sterile plastic-ware to avoid nuclease contamination. PCR reactions are extremely sensitive. The quality of the RNA is a critical factor in obtaining reliable and accurate results. To prevent RNA degradation and RNAse contamination, all sample and reagent preparations should be performed on ice unless noted otherwise. Work quickly and use RNAse-free tools and reagents (pipettes, pipette tips, dishes, tubes, etc). To avoid potential cross-over contamination of samples, change pipette tips frequently and after each sample. Proper aseptic laboratory practice should be followed to prevent microbial growth in the reagents.

Storage

This kit is stable for 6 months upon date of receipt under proper storage (-20°C). All of the reagents in the kit are provided in a ready-to-use format. Primers are supplied at optimal, validated concentrations. Primers and cDNA controls should be stored at -20°C until use. Aliquot and freeze primers and cDNA controls to avoid repeated freeze-thaw cycles.

Assay Instructions

It is assumed that the user will have already isolated high-quality RNA and reverse transcribed the RNA to cDNA using commercially available kits. The following PCR protocol uses Taq platinum DNA polymerase. If you are using a different DNA polymerase, follow the protocol provided by the manufacturer of the enzyme. PCR conditions have been optimized for a 25 µL reaction volume. The amount of input cDNAs have also been optimized for a 25 µL PCR reaction volume. The volumes for each component in the reaction mixture may be decreased or increased proportionally.

1. Thaw kit components (primers and cDNA controls) on ice.
2. Mix and briefly centrifuge each component vial. Place on ice.
3. Prepare PCR reaction mix for each primer set in a separate clean RNAse-free PCR tube.

	Per reaction
10X PCR reaction buffer*	2.5 µL
10 mM dNTPs	0.5 µL
50 mM $MgCl_2$*	0.75 µL
10 µM primer mix	0.5 µL
5X loading dye (optional)	5.0 µL
Taq platinum DNA polymerase*	0.2 µL
cDNA template (see chart below)	x µL
Sterile distilled water	To final 25 µL

* *The volumes of the 10X PCR reaction buffer, $MgCl_2$ and DNA polymerase should be based upon the instructions provided with the DNA polymerase enzyme.*

4. Add cDNA templates to individual PCR reaction mixtures. The amount of cDNA that is required will need to be determined empirically as there are variations in preparations along with different copy numbers of gene transcripts that are being probed. The following parameters are recommended as a guide to start the initial experiments. These parameters may need to be adjusted depending upon the quality of the samples.

Gene transcript being probed	cDNA template required	Volume required (using 10 ng/mL cDNA stock)	Volume Required (using 1 ng/mL cDNA stock)
GAPDH*	2 ng	-	2.0 µL
Nanog	25 ng	2.5 µL	-
Oct-4	10 ng	1.0 µL	-
AFP*	5 ng	-	5.0 µL
α-MHC	100 ng	10 µL	-
Pax-6	20 ng	2 µL	-

* Control cDNA for undifferentiated human ES cells (ES cDNA control) and for differentiated EBs (EB cDNA control) are provided in the kit at a 10 ng/µL concentration. For certain transcripts such as GADPH and AFP, the amount of cDNA templates required are very low. We thus recommend that the control cDNA be diluted 1:10. To make a 1 ng/mL diluted control cDNA sample, add 1 µL control cDNA to 9 µL sterile distilled water. Mix well by pipeting up and down. Centrifuge briefly to collect samples.

5. Add the appropriate amount of sterile distilled water to the reaction mixture so that the total volume is 25 µL.
6. Mix the reaction mixture thoroughly by pipeting up and down.,

7. Centrifuge PCR tubes briefly.
8. Transfer the reactions to a PCR machine and perform PCR using the following optimized cycling parameters:
   a. Initial denaturation at 94°C for 2 minutes
   b. 27 cycles of:
      94°C, 30 seconds
      55°C, 30 seconds
      72°C, 45 seconds
   c. Final extension at 72°C for 10 minutes

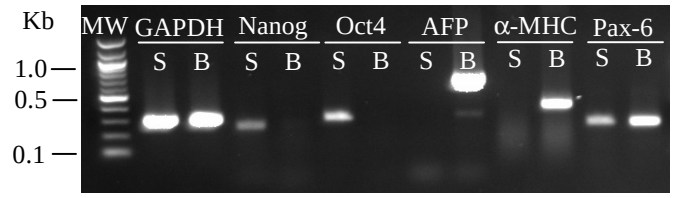
9. After the PCR reactions have terminated, the samples can be stored at 4°C. For long term storage, samples should be stored at -20°C.

10. Analysis of Results

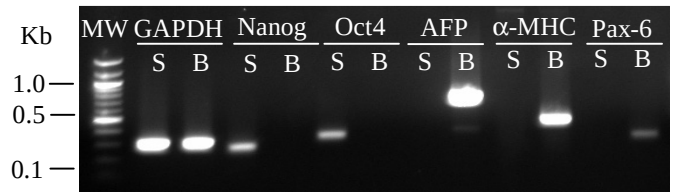
Following the PCR reaction, prepare a 1.5% agarose gel and directly load 10 to 25 µL of each PCR reaction mix to each well. Samples containing 5X loading dye can be loaded directly onto the gel. Although the reaction mix is compatible with glycerol-based loading dyes, it is not necessary to include additional loading dyes for analysis. Be sure to include a molecular weight marker with bands between 100 bp to 1000 bp in an adjacent well. The gel can be analyzed when the red dye has migrated to 2/3 distance from the loading point.

Results

(A) H9 human ES cells cultured in HEScGRO Medium (SCM021)
ES (S) and 30-day differentiated EB (B) cells



(B) H9 human ES cells cultured in KOSR Medium
ES (S) and 17-day differentiated EB (B) cells



(C) MEL-1 human ES cells (SCC020) cultured in KOSR Medium
ES (S) and 24-day differentiated EB (B) cells

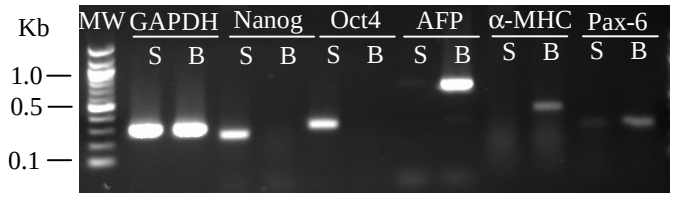
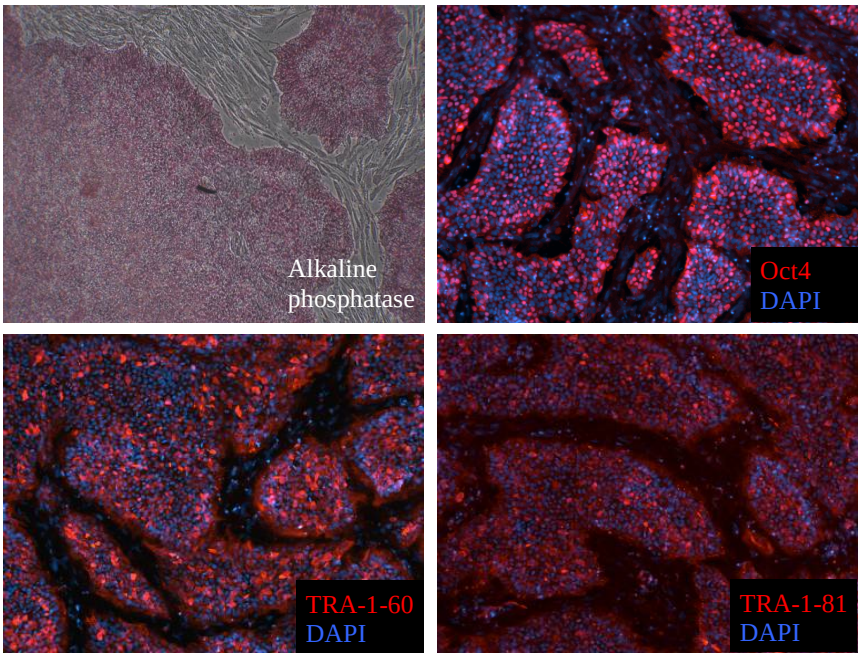


Figure 1. RT-PCR analyses display relative expression levels of gene transcripts that are commonly associated with pluripotent (Nanog and Oct-4) and differentiated states (AFP, α-MHC and Pax-6) of human ES cells. cDNAs were generated from undifferentiated H9 human ES cells cultured in Millipore's HEScGRO medium (**A**) or in KOSR medium (**B**) and differentiated as EBs for 30 days and 17 days respectively. cDNA was also generated from undifferentiated MEL-1 human ES cells that was cultured in KOSR medium and differentiated as EBs for 24 days (**C**). 10 μL of PCR samples were loaded in each well of the agarose gel. **S** denotes undifferentiated human ES cDNA and **B** denotes differentiated EBs.

The data indicates that the appropriate pluripotent markers, Nanog and Oct-4 are being expressed in different lines of human ES cells that have been cultured in different human ES cell media. Expressions of Nanog and Oct-4 are noticeably absent in differentiated EBs while differentiation markers for endoderm (AFP), mesoderm (α-MHC) and ectoderm (Pax-6) were significantly upregulated in the EBs.

(A)

H9 hES cells cultured in Millipore's HEScGRO medium for 20 passages



(B)

H9 EB cells cultured in HEScGRO medium and differentiated for 34 days

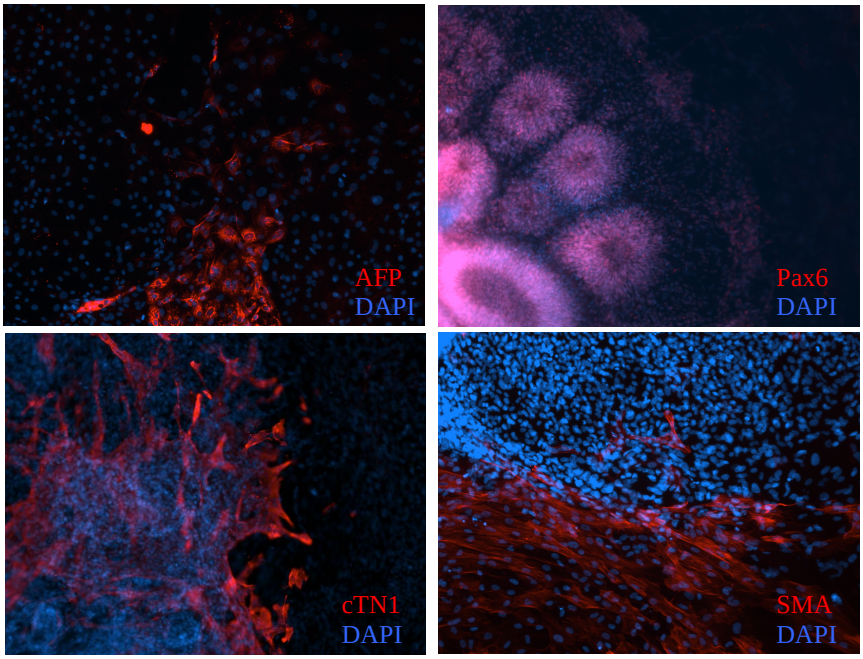


Figure 2. Immunofluorescence analysis of H9 human ES cells cultured in Millipore's HEScGRO Medium indicate that the appropriate pluripotent markers are being expressed (alkaline phosphatase, Oct-4, Tra 1-60 and Tra 1-81(A). H9 human ES cells differentiated as EBs for 34 days (B) can give rise to cells of endodermal (AFP), mesodermal (cTN1 and SMA) and ectodermal lineages (Pax-6). RT-PCR (Figure 1) and immunofluroescent (Figure 2) data are consistent with the fact that undifferentiated human ES cells are pluripotent and have the capacity to give rise to cells of the three embryonic germ layers.

Troubleshooting

Problem	Cause	Solution
No PCR band or poor yield	Poor cDNA quality and quantity	Repeat total RNA isolation using extreme caution to prevent possible nuclease contamination.
	Suboptimal PCR reagents	Check shelf life/storage conditions of PCR reagents; Replace with reagents from reliable sources.
Multiple bands in a single reaction	Sample or primer contamination	Replace tips frequently and between individual reactions to prevent cross-over contamination. Even trace levels of contaminating elements will affect final PCR results
Control PCR (GAPDH) appears to be uneven between reactions	RNA samples are not prepared under identical conditions; Determination of RNA concentration is not standardized and incorrect.	Prepare all samples simultaneously prior to cDNA synthesis. Synthesize cDNA under the exact same conditions where possible. If necessary, clean up cDNA after synthesis and standardize concentrations before PCR
	Genomic DNA contamination during RNA preparation	Treat RNA samples with high quality DNase I before determining the final concentration of the RNA and proceeding with cDNA synthesis
	Other RNA contamination	Final RNA concentration from ES cells grown on MEFs may be less accurate due to the contamination of mouse RNA. Remove feeders before RNA preparation or culture ES cells to high density to minimize contamination

Related Products

Millipore also provides additional reagents and kits for hESC related research:

- Alkaline Phosphatase Detection Kit (Catalog no.SCR004)
- Quantitative Alkaline Phosphatase ES Characterization Kit (Catalog no. SCR066)
- Human Embryonic Germ Layer Characterization Kit (Catalog no. SCR030)
- HEScGRO Medium for Human ES Cell Culture (Catalog no. SCM020, SCM021)
- MEL-1 Human Embryonic Stem Cell Line (Catalog no. SCC020)
- Pluripotent marker Oct-4 (Catalog no. MAB4401)
- Pluripotent marker TRA-1-60 (Catalog no. MAB4360)
- Pluripotent marker TRA-1-81 (Catalog no. MAB4381)
- Neuronal cell marker Pax6 (Catalog no. MAB5409)
- Cardiomyocyte marker cTn1 (Catalog no. MAB1691)
- Smooth muscle actin marker SMA (Catalog no. CBL171).

References

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2. Liang J, Wan M, Zhang Y, Gu P, Xin H, Jung SY, Qin J, Wong J, Cooney AJ, Liu D, Songyang Z. (2008) Nanog and Oct4 associate with unique transcriptional repression complexes in embryonic stem cells.Nat Cell Biol.10(6):731-9.

3. International Stem Cell Initiative, Adewumi O, Aflatoonian B, Ahrlund-Richter L, Amit M, Andrews PW, Beighton G, Bello PA, Benvenisty N, Berry LS, Bevan S, Blum B, Brooking J, Chen KG, Choo AB, Churchill GA, Corbel M, Damjanov I, Draper JS, Dvorak P, Emanuelsson K, Fleck RA, Ford A, Gertow K, Gertsenstein M, Gokhale PJ, Hamilton RS, Hampl A, Healy LE, Hovatta O, Hyllner J, Imreh MP, Itskovitz-Eldor J, Jackson J, Johnson JL, Jones M, Kee K, King BL, Knowles BB, Lako M, Lebrin F, Mallon BS, Manning D, Mayshar Y, McKay RD, Michalska AE, Mikkola M, Mileikovsky M, Minger SL, Moore HD, Mummery CL, Nagy A, Nakatsuji N, O'Brien CM, Oh SK, Olsson C, Otonkoski T, Park KY, Passier R, Patel H, Patel M, Pedersen R, Pera MF, Piekarczyk MS, Pera RA, Reubinoff BE, Robins AJ, Rossant J, Rugg-Gunn P, Schulz TC, Semb H, Sherrer ES, Siemen H, Stacey GN, Stojkovic M, Suemori H, Szatkiewicz J, Turetsky T, Tuuri T, van den Brink S, Vintersten K, Vuoristo S, Ward D, Weaver TA, Young LA, Zhang W. (2007).Nat Biotechnol. Characterization of human embryonic stem cell lines by the International Stem Cell Initiative.25(7):803-16

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Cat No. SCR063

06/2010
Revision B: SCR063MAN