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Running head: *CCND1*amplification

Abstract
*CCND1*located in 11q13 encodes the G1-S regulatory protein, cyclin D1, is frequently amplified in various types of cancers, and is an attractive potential therapeutic target. Multiplex ligation-dependent probe amplification (MLPA) is a new, high-resolution method for the detection gene amplification of numerous genes including *CCND1* in small amounts of DNA fragments derived from formalin-fixed paraffin-embedded material in a single reaction. This approach is, however, based on PCR and averages many different cells, so validation by morphological methods such as FISH is theoretically mandatory. Here we described detection of *CCND1* gene copy number variations by commercially available MLPA kits and FISH using a bacterial artificial chromosome (BAC) probe.

Key words: formalin-fixed paraffin-embedded material, commercially available MLPA kits, BAC probe

1. Introduction

Under normal circumstances, growth factor signaling leads to the expression of cyclin D1 and its complexing with cyclin-dependent kinase 4 (CDK4) or CDK6. Following accumulation of active cyclin D1/CDK4 or CyclinD1/CDK6, CDK2 in combination with cyclin E then accumulates to facilitate the transition from G1 to S phase by phosphorylation of downstream targets, including the tumor suppressor RB [1]. CCND1 is amplified in various types of cancer such as head and neck, endometrium, pancreas, breast and stomach. [2] Cyclin D1 are generally regarded as difficult to target directly with therapies, as they lack intrinsic enzymatic activity and are intracellular. Thus, their functionality may most readily be targeted via their partner kinases, CDK4 or CDK6 [3]. In molecularly targeted therapies, establishing feasible screening methods to identify eligible patients is crucial. Compared to SNP, aCGH and next-generation

sequencing techniques, MLPA is a relatively cheap, easy-to-perform method that allows simultaneous detection of multiple gene copy-number aberrations in small amounts of DNA fragments derived from formalin-fixed material [4]. This approach is, however, based on PCR and averages many different cells, so validation by morphological methods such as FISH is theoretically mandatory. [5]

2. Materials

2.1 MLPA

1. MLPA kits containing probes enumerating *CCND1* copy number are commercially available from MRC-Holland (Amsterdam, The Netherlands): P175-A2 Tumor-Gain®, P458-B1 Gastric Cancer®, P078-C1 Breast cancer®)
2. H_2O: distilled water is filtered with Water Purification System of Millipore and autoclaved.
3. 1M NaSCN: dissolve 81g sodium thiocyanate in 1L of H_2O.
4. TE bufferes: Mix Tris-Cl (1M) with an appropriate pH and EDTA (0.5M) pH 8.0, and adjust the final concentrations of Tris-Cl 10mM and EDTA 1mM. Tris-Cl (1M): dissolve 121.1g of Tris base in 800ml of H_2O. Adjust to the desired pH by adding concentrated HCl . Adsjust the volume of the solution to 1L with H_2O. EDTA (0.5M) pH 8.0: Add 186.1g of disodium EDTA· $2H_2O$ to 800ml of H_2O. Adjust pH to 8.0 with NaOH,
5. Proteinase K: PCR grade, Roch (Basel, Switzland)
6. 20xSSC: Dissolve 175.3g NaCl, 88.2g *tri*-sodium citrate dehydrate in 800ml distilled water, adjust the pH to7.0 with 1*N* HCl, and adjust to 1L with H_2O.

2.2 FISH

1. LB medium: Dissolve 10g bacto-tryptone, 5g bacto-yeast extract, and 10g NaCl in 950ml distilled water, adjust the pH to 7.0 with 5 N NaOH. Adjust the volume of the solution to 1 liter with distilled water. Sterilize by autoclaving.
2. P1 buffer®, P2 buffer® and P3 buffer®, QIAGEN tip 20®, Buffer QBT®, Buffer QC®from Qiagen plasmid Mini kit® : Qiagen (Hilden, Germany)
3. Kimwipes® : Kimberly-Clark Corporation (Neenah, Wis, USA)
4. Nick translation kit: Abbott Laboratories (Abbott Park, IL, USA)
5. Cot 1 DNA (1 μg/μl) : Invitrogen (Carlsbad, CA, USA)
6. Human placental DNA (1 μg/μl): Sigma (St. Louis, MO, USA)
7. E. coli tRNA (1 μg/μl) : Sigma
8. MAS-coated glass slides: Matsunami, (Tokyo, Japan)

9. RNase A (100 mg/ml): Qiagen
10. Thermo Brite: Abbott Laboratories (Abbott Park, IL, USA)
11. 0.1% g/ml Protease solution® : Abbott Laboratories (Abbott Park, IL, USA)
12. 100% formamide: specially prepared reagent, Nachalai tesque (Kyoto, Japan)
13. NP-40: igepal® CA-680 (Sigma)
14. Locus Specific Identifier DNA probe (LSI): Abbott Laboratories (Abbott Park, IL, USA)
15. DAPI-II® / Anti fade® solution: Abbott Laboratories (Abbott Park, IL, USA)
16. Pretreatment Solution®: Abbott Laboratories (Abbott Park, IL, USA)

3. Methods

3.1. MLPA

DNA is extracted from FFPE tissue according to the manufacturer's protocol (Protocol DNA extraction from FFPE tissue, MRC-Holland) with some revision.

1. Cut three to four 6 μm sections and put them on uncoated slides. Several adjacent 4 μm sections on MAS-coated slides are used for HE, FISH and IHC
2. Trim the examined area referring the adjacent HE section, unnecessary areas are removed by razor blade.
3. Heat the slides at 75 °C for 15 min on hot plate to melt the paraffin
4. De-paraffinate in Coplin jar consectively in 100% xylene (HistClear) two times 5min each, in 100% EtOH two times for 30 sec each, then wash in water.
5. Remove excess water from the glass slide
6. Drop 50-100μl 1M NaSCN on the section and leave for a few minutes.
7. Peel the section from the glass slide by a spatula, and transfer to 1.5ml Eppendorf's tube with 1M NaSCN. Incubate at 37 °C, overnight.
8. Centrifuge 15,000rpm by a microcentrifuge for 10min. Discard supernatant. After adding distilled water tap and leave for a few minutes
9. Centrifuge 15,000rpm by a microcentrifuge for 10min. Discard supernatant and adding TE buffer pH7.2. Tap and leave for a few minutes.
10. Centrifuge 15,000rpm by a microcentrifuge for 10min. Discard supernatant. Add 50-100μl of proteinase K 0.5mg/ml and Incubate at 55 °C overnight.
11. Inactivate proteinase K by incubation at 80 °C for 20min.
12. Centrifuge 15,000rpm by a microcentrifuge for 10min. Transfer the supernatant to new tube.

13. Measure OD 280/260 by NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MAS USA) and adjust DNA concentration of 30-40 ng/μl by TE pH8.2

DNA denaturation, hybridization reaction, ligation reaction and PCR reaction are done thermocycler program for the MLPA reaction exactly according to the 'MLPA DNA Protocol version MDP-005; last revised on 22 SEPT 2014'(MRC-Holland)

Reaction product is separated by capillary sequencer ABI-310 (Applied Biosystems, foster City, CA, USA) . The DATA are analyzed by Coffalyser. (MRC-Holland)

3.2 FISH

For designing FISH probes, generally gene specific BAC probes are searched using UCSC Genome Browser or NCBI map viewer, and the LB agars stabbed with E. Coli carrying BAC probe are available through BACPAC resources (Oakland, CA, USA). In our previous studies we used RP11-300I6 (69, 226, 211-69,387,715) which covers *CCND1* (chromosomal position: 69,228,876-69,242,171) [6] . Fluorescein-labelled ready-for-use probes for *CCND1*are also commercially available from Abbott Laboratories (Abbott Park, IL, USA)

Extraction and purification of BAC DNA

1. Spread bacterial suspension by streaking surface of the agar plate with an appropriate antibiotics.
2. After overnight-culture of the plate at 37℃, pick up a single colony and transfer it to 3ml of LB medium with antibiotics in 50ml tube, and incubate at 37℃for 8h in 160 rpm.
3. Transfer 50μl of the culture medium to 50ml LB medium with antibiotics in 300ml flask and incubate at 37℃overnight by shaking at 160rpm.
4. Transfer the culture medium to 50ml tubes and centrifuge at 2,200xg for 10 min at 4℃.
5. Discard the supernatant. Add 6 μl of RNase dissolved in 6ml of P1 buffer® and mix it by Vortex.
6. Add 6ml of P2 buffer®. Invert 4-5 times to mix and stand for 5min.
7. Add 6ml of P3 buffer®. Invert 4-5 times to mix and stand on ice 15 min repeat this three times.
8. Add 130 μl of chloroform and transfer to centritube. Centrifuge at 24,000xg at

4℃ for 20 min.

9. Filter the solution by filter paper. Add 18 ml of isopropyl alcohol to the supernatant and mix it lightly
10. Centrifuge at 24,000xg at 4℃ for40min-1h.
11. Discard the supernatant. Stand the tube in an inverted position on a paper towel to allow all the fluid to drain away. Remove any drops of fluid adhering to the wall of the tube by Kimwipe®. Be careful not to dry the pellet completely.
12. Dissolve the precipitate with 300μl of TE buffer (pH7.0).
13. Equilibrate a QIAGEN-tip 20® by applying 1ml Buffer QBT®, and allow column to empty by gravity flow.
14. Apply the supernatant from step 12 to the QIAGEN-tip® and allow it to enter the resin by gravity flow.
15. Wash the QIAGEN–tip® with 2ml Buffer QC® two times. Allow Buffer QC® to move through the QIAGEN-tip® by gravity flow.
16. Elute DNA with 0.8ml Buffer QF® prewarmed to 65℃ onto a clean 2ml vessel.
17. Precipitate DNA by adding 0.56 ml (0.7 volume) room-temperature isopropanol to the eluted DNA and mix. Centrifuge at >15, 000rpm for 30 min at 4℃. Carefully decant the supernatant.
18. Wash the DNA pellet with 1ml room-temperature 70% ethanol and centrifuge at >15,000rpm by a microcentrifuge for 10 min. Carefully decant supernatant
19. Air-dry pellet by speed vacume for 5-8min.
20. Resolve the pellet by 20μl of TE buffer (pH8.5).
21. Measure the DNA content and adjust the concentration of 1 μg/μl.

Nick translation procedure

1. Place a microcentrifuge tube on ice and allow the tube to cool.
2. Add these components (Nick translation Kit®) to the tube in the order listed. Briefly centrifuge and vortex the tube before adding the enzyme (last component). This procedure label 1μg of extracted BAC DNA which is enough for ten FISH experiments (one target area equal to 22x22mm).

			Order of adding
	dH_20	16.5 μl	1
1 μg/μl	BAC DNA	1 μl	2
0.2 mM	fluorescence-labelled dUTP	2.5 μl	3
0.1 mM	dTTPs	5 μl	4
0.1 mM	dNTP mix	10 μl	5

10x nick translation buffer buffer	5 µl	6
Nick translation Enzyme	10 µl	7

Briefly centrifuge and votex the tube

3. Incubate at 15℃for 5-10h.
4. Stop the reaction by heating in a 70℃water bath for 3 min. Chill on ice.
5. Ethanol precipitation: Add these components to the tube and vortex briefly.

Reaction solution of Step 2	50 µl
Cot 1 DNA (1 µg/µl)	10 µl
Human placental DNA (1 µg/µl)	20 µl
E. coli tRNA (1 µg/µl)	10 µl
dH_2O	30 µl

6. Add 12µl (1/10v) 3M sodium Acetate and 300 µl (2.5 v) of 100% ethanol. Briefly vortex and centrifuge. Keep at -80℃ for 1h Centrifuge 15,000rpm by a microcentrifuge at 4℃ for30min.
7. Discard the supernatant. Air-dry pellet by speed vacume for 5-8min. Suspend the pellet in 10µl of dH_20.
8. Keep in freezed in dark.

Dual-color FISH on formalin-fixed and paraffin-embedded specimens (Note 1)

1. 4µm-thick sections were placed onto the MAS-coated glass slides®. Mark hybridization areas with a diamond-tipped scriber on the bottom of the specimen slide.
2. Deparaffinize the section by three successive 10-min washes in in Histo-Clear, followed by three 5-min washes in absolute ethanol.
3. Air-dry the slides and incubate in 0.2 N HCl at room temperature for 20min.
4. Wash slides in distilled water for 3 min.
5. Wash slides in 2x SSC for 3 min.
6. Incubate slides in a glass Coplin jar containing 40 ml of the Pretreatment Solution® at 80℃for 30 minutes, followed by 1-min wash in distilled water and two 5-minutes wash in 2x SSC.
7. Incubate slides in RNase A (100 µg/ml in 2x SSC) at 37℃ for 30min using ThermoBrite®.

8. Wash slides in 2x SSC two times for 5 minute each.
9. Incubate slides in 100µl of 5 mg/ml Protease solution® at 37℃ for 30min using ThermoBrite®.
10. Wash slides in 2x SSC two times for 5 min each
11. Rinse slides in 10% buffered formalin in PBS at RT for 10min
12. Wash slides in 2x SSC two times for 5 min each.
13. Dehydrate the slide in successive rinses in 70%, 85%, 100% ethanol for 2min each.
14. Allow slides to dry. Aided by cool air flow.
15. Probe preparation: Add these components to the Eppendorf tube. This preocedure label 10µl of the probe solution enough for a FISH experiment (one target area equal to 22x22mm).

Gene-specific probe	1 µl
CEP 11probe®	1 µl
dd$H_2$0	1 µl
LSI/WCP hybridization buffer®	7 µl
	10 µl

Briefly centrifuge and vortex the tube.

16. Apply probe and seal cover glass by paper bond.
17. Denature and hybridization: heat slides at 75℃ for 5min and incubate slides at 37℃ over-night.
18. Posthybridization washing: wash slides in 40 ml of 50% formamide / 2x SSC in Coplin jar at 45℃ three times for 10 min each. (100% formamide 20ml / 4xSSC 20ml)
19. Wash slides in 2xSSc, at 45℃ for 10 min
20. Rinse the slides in 40 ml of 0.1% NP-40 / 2xSSC, at 45℃ for 10 min
    10% NP-40 o.4 ml/ 2xSSC, 39.6ml
21. Rinse slides in 2x SSC at room temperature two times, 5 min each
22. After air dry add DAPI-II® / Anti fade® solutiom, and cover with cover slip, Put slides in suitable boxes, which should be kept ay -20℃ before observation.
23. Examine slides with a fluorescence microscope (Olympus) equipped with Triple Bandpass Filter sets® (Abbott) for DAPI II, SpectrumOrange®, and SpectrumGreen® (Note 2)

4. Notes

1. To detect a numerical aberration of a gene, dual-color FISH was applied: a centromeric probe was used as a reference probe to assist in distinguishing real gene amplification from an increased gene number resulting from chromosomal polysomy at which the gene is located. Some fluorescence-labelled centromeric specific probes are commercially available.

2. The critical step in FISH using paraffin-embedded tissue is the removal of nuclear protein by enzymatic digestion. The optimal digestion condition may be modified to accommodate each section, because fixation conditions were different for various individual specimens. If FISH signals look blurred in white cloudy nuclei instead of DAPI-positive blue nuclei, additional digestion may remarkably improve the image. This can be done as follows:
    1. Remove the coverslip in 2xSSC.
    2. Heat-denature the probes: incubate slides in 50% formamide / 2x SSC at 75℃ for 15min using Coplin jar.
    3. Wash slides in 2xSSC two times for 5 min each
    4. Go to Step 9 and digest for additional time of 20-60min and follow Steps
3. Although we mention a specific supplier for kits or reagents, there may be acceptable products from other suppliers.

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