付表９（つづき）

6112803	○○○○○○○○	○○○○○○○○○○○○○○	○○○○○○	□×○○○	95.8%
6112804	○○○○○○○○	○○○○○○○○○○○○○○	○○○□×△	□○×○○	90.1%
6112805	○○○○○○○○	○○○○○○○○○△○○	△○○○×××	××××○×	78.1%
6112806	○○○○○○○○	○○○○○○○△○○○○	○○○□××	×▲×○○	82.8%
6112901	○○○○○○○○	○○○○○○○○○▲△○	○○○□××	▲△×○○	82.3%
6112902	○○□○○○○○	○○○○○○○○○△△△	○○○×△□	○○□○○	87.5%
6112903	○○○○○○○□	○○○○○○○○○○□○	○○○×○○	○△□○○	92.2%
6112904	○○○○○○○○	○○○○○○○○○○○○	○○○○△△	□○▲○○	93.8%
6112905	○○○○○○○○	○○○○○○○○○○○○	○○○×□□	○○×○○	91.7%
6112906	○○○○○○○○	○○○○○○○○○△□○	○○○▲×△	□×□○○	88.5%
6113001	○○○○○○○○	○○○○○○○○○○○○	○○○○▲○	○○□○○	96.9%
6113002	○○○○○○○○	○○○○○○○○△○△□○	□○○▲×□	△□△○○	84.4%

付表１０

	C1	2	3	4	5	6	7	8	T1	2	3	4	5	6	7	8	9	10	11	12	13	L1	2	3	4	5	6	S1	2	3	4	5	除去率
6120101	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	×	□	△	×	○	○	89.6%
6120102	□	○	○	○	□	○	○	○	○	○	○	○	○	○	○	○	○	△	○	○	○	○	○	○	○	○	○	○	×	△	○	○	91.7%
6120103	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	△	○	○	○	○	▲	△	□	□	□	△	○	○	89.1%
6120104	○	○	○	○	□	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	×	△	□	○	□	▲	○	○	89.1%
6120105	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	△	○	○	○	△	○	○	95.8%
6120106	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	○	○	△	○	○	○	×	×	△	□	○	○	○	○	88.0%
6120107	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	□	○	○	○	○	□	×	×	▲	○	○	○	○	○	88.0%
6120108	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	△	○	○	○	○	○	○	□	△	×	▲	▲	×	○	○	83.8%
6120401	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	△	○	○	○	○	○	×	▲	×	□	▲	□	○	○	84.9%
6120402	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	□	○	○	○	○	○	□	○	○	△	□	○	○	94.3%
6120403	○	○	○	○	▲	○	○	○	○	○	○	○	○	○	○	○	○	○	○	▲	○	○	○	○	○	△	□	○	□	×	○	○	89.1%
6120404	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	□	○	○	△	○	○	△	△	○	○	□	○	○	91.7%
6120501	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	○	○	○	○	○	×	△	□	○	○	○	○	○	93.2%
6120502	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	○	○	○	○	○	○	○	○	□	×	×	□	△	×	○	○	85.4%
6120601	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	○	▲	○	○	○	○	○	▲	×	×	×	×	×	×	×	72.9%
6120602	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	○	○	○	○	○	○	□	×	×	×	×	○	○	84.9%
6120701	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	×	○	△	▲	×	▲	○	○	88.0%
6120702	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	▲	○	○	○	○	○	○	▲	▲	○	○	□	□	○	○	91.7%
6120703	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	×	▲	○	○	○	□	○	○	93.8%
6120801	○	○	○	○	○	△	○	○	○	○	○	○	○	○	○	○	□	□	×	○	○	○	○	○	△	△	□	□	○	×	△	○	83.3%
6120802	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	×	△	○	○	○	□	○	○	94.2%
6120803	○	□	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	○	○	○	△	○	○	▲	×	△	×	△	□	○	83.8%
6120804	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	○	○	□	△	○	○	○	○	×	×	×	×	×	○	○	80.7%
6120805	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	○	×	□	□	△	○	○	91.7%
6120806	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	△	△	○	○	○	○	○	×	×	×	□	○	□	○	○	83.8%
6121101	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	▲	△	△	△	○	○	○	□	▲	△	▲	×	▲	○	○	79.7%
6121201	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	×	×	○	○	○	×	○	○	90.6%
6121301	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	△	×	×	△	△	○	○	88.0%
6131302	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	□	×	×	□	○	○	○	93.2%
6121401	○	○	○	○	○	○	○	▲	△	▲	▲	○	○	○	○	○	▲	▲	○	○	○	○	○	○	×	×	×	△	○	▲	○	○	75.0%
6121501	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	○	○	○	○	×	×	△	○	×	×	○	○	84.9%
6121502	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	○	×	△	○	○	○	○	○	×	□	○	□	□	×	○	○	84.4%
6121503	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	▲	○	○	○	□	△	○	○	95.3%
6121504	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	▲	△	□	○	○	□	○	×	91.2%
6121505	○	△	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	○	○	○	○	○	□	△	○	○	○	○	○	○	94.3%
6121506	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	○	○	○	○	○	△	△	○	○	○	×	○	○	92.7%
6121801	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	○	○	○	○	○	□	○	×	×	×	▲	○	○	86.5%
6121802	□	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	×	○	○	○	○	▲	×	△	▲	×	×	○	○	80.7%
6121803	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	×	▲	□	□	×	○	○	89.6%
6121901	○	○	○	○	○	□	○	○	○	○	○	○	○	○	○	○	○	○	○	□	△	▲	○	○	×	×	○	○	○	×	○	○	84.9%
6121902	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	○	○	○	△	×	×	×	×	○	×	81.8%
6121903	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	○	○	○	○	○	○	□	△	○	○	○	○	○	96.3%
6121904	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	×	○	○	○	△	○	×	▲	×	×	□	▲	○	○	79.7%
6122001	○	○	○	○	○	○	○	□	○	△	○	○	○	○	○	○	○	○	□	○	○	○	○	○	×	×	▲	△	△	▲	○	○	82.8%
6122002	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	□	○	○	○	□	△	×	△	×	▲	×	○	○	82.3%
6122101	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	▲	○	○	□	▲	△	×	□	○	○	87.5%
6122102	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	×	△	×	○	△	○	○	89.1%
6122103	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	○	○	○	○	○	○	○	○	×	×	○	□	△	○	○	90.1%
6122104	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	□	□	○	○	○	×	93.8%
6122201	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	○	○	○	○	○	○	□	×	×	×	▲	○	×	82.8%
6122202	○	○	○	○	○	△	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	○	△	×	×	○	○	89.1%
6122501	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	×	×	▲	×	□	×	○	○	84.4%
6122502	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	△	○	□	○	×	○	×	89.6%

付表１１

	C1	2	3	4	5	6	7	8	T1	2	3	4	5	6	7	8	9	10	11	12	13	L1	2	3	4	5	6	S1	2	3	4	5	除去率
7010501	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	○	○	○	○	○	○	○	○	○	▲	△	○	○	94.8%
7010502	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	▲	□	□	△	○	○	▲	△	○	○	○	×	○	○	86.5%
7010503	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	▲	○	×	○	○	×	×	×	×	×	○	○	80.2%
7010504	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	○	○	○	○	○	○	×	△	△	×	○	○	85.9%
7010601	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	▲	○	○	○	○	○	○	97.9%
7010602	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	○	○	○	○	○	×	×	×	×	○	△	○	○	84.4%
7010603	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	×	×	×	○	×	△	○	○	85.9%
7010604	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	×	▲	□	○	○	□	○	○	92.7%
7010901	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	△	□	○	△	○	○	□	□	○	▲	×	▲	○	○	85.4%
7010902	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	▲	▲	×	□	○	○	90.6%
7011001	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	○	○	○	○	○	○	▲	○	△	×	△	□	○	○	89.6%
7011002	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	○	○	○	○	○	×	▲	△	□	△	△	○	○	88.0%
7011003	○	○	○	○	○	○	○	▲	○	△	○	○	○	○	○	○	○	□	□	○	○	○	△	○	□	×	×	▲	×	▲	○	○	78.1%
7011004	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	○	○	○	○	□	○	×	94.8%
7011101	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	○	○	○	○	○	○	○	○	×	○	○	○	○	△	○	○	93.8%
7011201	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	○	○	○	○	○	△	□	×	○	△	△	○	○	89.6%
7011202	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	□	○	□	○	○	○	○	×	×	▲	△	▲	○	○	84.9%
7011501	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	△	○	○	○	○	○	□	×	□	□	□	△	○	○	88.3%
7011502	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	○	○	○	○	○	○	△	×	×	×	×	×	○	○	81.8%
7011503	○	○	○	○	○	○	▲	○	○	○	○	○	○	○	○	○	▲	○	○	○	○	○	○	○	○	×	○	○	○	□	○	×	88.5%
7011504	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	○	○	○	○	○	○	×	×	□	○	○	△	○	○	90.1%
7011601	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	□	○	□	○	○	○	○	○	×	×	○	×	△	△	○	○	84.4%
7011602	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	×	▲	○	▲	▲	○	○	89.1%
7011603	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	△	×	△	×	×	○	○	86.5%
7011701	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	×	□	×	○	×	□	○	○	88.6%
7011702	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	×	×	×	○	○	×	○	○	87.5%
7011703	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	○	□	○	○	○	○	○	□	×	×	×	○	□	×	○	83.3%
7011704	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	□	○	○	○	○	○	○	○	□	□	□	×	○	○	91.2%
7011901	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	○	○	○	○	□	○	○	□	▲	○	□	○	○	93.3%
7011902	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	○	○	○	○	○	▲	×	×	▲	×	×	○	○	81.8%
7012201	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	□	○	○	○	○	○	×	×	▲	△	○	□	○	○	87.0%
7012202	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	▲	□	○	△	○	○	×	×	△	□	□	×	○	○	81.3%
7012301	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	○	○	○	○	○	△	▲	○	○	○	○	○	○	94.8%
7012302	○	○	○	○	○	○	○	□	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	▲	○	×	×	○	○	89.6%
7012401	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	×	×	△	△	×	□	○	○	86.5%
7012402	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	×	□	×	□	□	○	○	90.6%
7012403	○	○	○	○	○	○	○	○	○	○	○	○	○	△	○	○	○	○	○	○	○	○	○	○	□	○	○	○	□	□	○	○	95.3%
7012501	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	○	○	○	○	○	▲	×	×	×	×	×	△	○	○	79.7%
7012502	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	○	○	○	○	○	×	○	□	○	○	□	○	×	90.1%
7012503	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	○	×	×	×	▲	△	▲	○	○	83.3%
7012601	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	▲	○	○	×	▲	▲	×	×	○	○	○	82.8%
7012602	○	○	○	○	○	□	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	○	×	×	○	×	○	○	88.6%
7012603	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	×	▲	×	▲	×	○	○	86.5%
7012901	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	×	△	○	○	○	○	○	□	□	○	○	□	○	○	○	92.2%
7012902	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	▲	△	□	○	○	○	×	×	○	○	○	□	○	○	86.5%
7012903	○	○	□	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	×	△	□	○	△	○	○	90.6%
7012904	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	○	○	○	○	○	○	▲	×	○	○	△	×	○	○	89.1%
7013001	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	○	○	○	○	○	○	×	×	×	×	▲	△	○	○	82.3%
7013101	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	×	×	○	○	×	△	○	○	87.5%
7013102	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	○	○	○	○	○	○	×	×	○	○	□	○	○	91.2%

付表１２

	C1	2	3	4	5	6	7	8	T1	2	3	4	5	6	7	8	9	10	11	12	13	L1	2	3	4	5	6	S1	2	3	4	5	除去率
7020101	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	▲	○	○	○	○	○	○	○	△	□	○	▲	○	○	93.2%
7020201	○	○	○	○	○	○	○	▲	○	○	○	○	○	○	○	○	○	○	▲	○	○	○	○	○	□	×	○	▲	×	×	○	○	83.3%
7020202	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	□	○	□	□	○	○	×	×	○	○	89.6%
7020501	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	×	○	□	▲	▲	▲	×	×	▲	○	×	78.1%
7020502	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	○	▲	×	×	×	×	×	□	○	○	79.7%
7020503	○	○	○	○	○	○	○	□	○	○	○	○	○	○	○	○	○	○	○	○	▲	○	○	○	○	△	□	△	□	□	○	○	90.6%
7020601	○	○	○	○	○	○	○	○	□	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	▲	□	△	▲	○	○	○	92.2%
7020701	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	○	○	○	○	△	×	○	△	○	○	×	○	○	89.6%
7020702	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	×	□	○	○	○	○	○	○	95.8%
7020703	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	×	□	○	○	○	○	×	×	×	×	△	△	○	×	77.1%
7020801	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	×	×	□	□	▲	×	○	○	86.5%
7020802	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	×	▲	▲	▲	△	○	○	89.1%
7020803	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	×	×	○	○	○	□	○	○	92.7%
7020901	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	×	▲	○	○	○	□	○	○	93.8%
7020902	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	×	×	×	×	△	×	○	○	82.8%
7020903	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	○	△	○	□	▲	○	○	94.3%
7021301	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	×	×	×	▲	△	×	○	○	83.8%
7021302	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	▲	□	□	□	×	×	○	○	88.5%
7021401	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	○	○	○	○	○	○	□	□	○	○	○	○	○	96.9%
7021402	○	○	○	○	○	○	○	○	○	○	○	○	□	○	○	○	○	○	○	○	○	○	○	○	○	□	×	×	□	□	○	○	89.6%
7021501	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	×	○	×	○	□	×	○	○	89.6%
7021601	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	×	×	×	×	○	○	87.5%
7022001	○	○	○	○	○	○	○	□	○	○	○	○	○	○	○	□	○	×	△	×	○	○	▲	○	×	▲	×	□	□	△	○	○	76.1%
7022002	○	○	○	○	○	○	○	□	△	△	○	△	○	○	○	○	○	△	▲	▲	○	□	○	○	×	×	×	▲	□	□	○	○	74.0%
7022003	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	▲	○	▲	△	□	△	○	○	91.7%
7022004	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	▲	×	○	○	▲	□	○	×	88.5%
7022101	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	○	○	98.4%
7022102	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	▲	×	□	○	×	90.6%
7022103	○	○	○	○	○	○	○	○	○	□	○	○	○	○	□	○	○	○	○	○	○	○	○	○	×	□	□	△	△	×	○	○	86.5%
7022104	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	×	×	×	▲	×	×	○	×	79.2%
7022301	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	○	○	○	○	○	□	□	×	○	△	×	○	○	89.1%
7022302	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	△	○	○	○	△	○	×	93.8%
7022303	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	×	▲	×	×	×	○	○	84.4%
7022601	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	▲	○	○	○	○	○	×	▲	○	○	□	□	○	○	90.6%
7022602	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	×	×	×	△	△	×	○	○	84.4%
7022603	○	○	○	○	○	○	○	○	○	○	○	○	○	△	○	○	○	○	□	○	○	○	○	○	△	×	▲	×	×	▲	○	○	82.3%
7022701	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	×	×	×	×	×	□	○	○	83.3%
7022702	○	○	○	○	○	○	○	○	○	○	□	○	○	○	○	○	○	○	□	○	○	○	○	○	×	×	×	×	○	△	○	×	80.8%
7022703	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	□	□	○	○	○	○	○	▲	□	△	×	×	○	○	87.0%
7022801	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	▲	○	○	△	○	○	96.3%
7022802	○	○	○	○	○	○	○	□	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	×	□	×	▲	□	□	○	○	87.5%

別添 5

研究成果の刊行に関する一覧表

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Kashida, Y., Takahashi, A., Moto, M., Okamura, M., Muguruma, M., Jin, M., Arai, K., Mitsumori, K.	Gene expression analysis in mice liver on hepatocarcinogenesis by flumequine.	Arch. Toxicol.	80	533-539	2006
Moto, M., Umemura, T., Okamura, M., Muguruma, M., Ito, T., Jin, M., Kashida, Y., Mitsumori, K.	Possible involvement of oxidative stress in dicyclanil-induced hepatocarcinogenesis in mice.	Arch. Toxicol.	80	694-702	2006
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Lee, K-Y, Shibutani, M., Inoue, K., Kuroiwa, K., U, M., Woo, G-H., Hirose, M.	Methacarn fixation – Effects of tissue processing and storage conditions on detection of mRNAs and proteins in paraffin-embedded tissues.	Anal. Biochem.	351	36-43	2006
Shibutani, M., Lee, K-Y, Igarashi, K., Woo, G-H., Inoue, K., Nishimura, T., Hirose, M.	Hypothalamus region-specific global gene expression profiling in early stages of central endocrine disruption in rat neonates injected with estradiol benzoate or flutamide.	Dev. Neurobiol.	67(3)	253-269	2007

Umemura, T., Kanki, K., Kuroiwa, Y., Ishii, Y., Okano, K., Nohmi, T., Nishikawa, A., Hirose, M.	*In vivo* mutagenicity and initiation following oxidative DNA lesion in the kidneys of rats given potassium bromate.	Cancer Sci.	97	829-835	2006
Kuroiwa, Y., Umemura, T., Nishikawa, A., Kanki, K., Ishii, Y., Kodama, Y., Masumura, K., Nohmi, T., Hirose, M.	Lack of *in vivo* mutagenicity and oxidative DNA damage by flumequine in the livers of *gpt* delta mice.	Arch. Toxicol.	81	63-69	2007

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Methacarn Fixation for Genomic DNA Analysis in Microdissected Cells

Makoto Shibutani and Chikako Uneyama

Summary

We have found methacarn, a non-crosslinking protein-precipitating fixative, to be useful for the analysis of DNA from microdissected specimens of wax-embedded tissue. In this chapter, we present the procedure regarding genomic DNA analysis in methacarn-fixed wax-embedded microdissected rat tissue. Using nested polymerase chain reaction (PCR), and a rapid extraction procedure, fragments of DNA up to 2.8 kb in size can be amplified from a 1 × 1 mm area of a 10-μm-thick tissue section. Target fragments of about 500 bp can be amplified from a single cell, but 10–20 cells are necessary for practical detection by nested PCR. Although tissue staining with hematoxylin and eosin inhibits the PCR, amplification of about 500-bp fragments is successful with 150–270 cells by single-step PCR. Immunostaining results in a substantial decrease of yield and degradation of extracted DNA. However, even after immunostaining, fragments of about 180 bp can be amplified with 150–270 cells by single-step PCR. These features demonstrate the suitability of methacarn-fixed wax-embedded tissue for practical genomic DNA analysis in terms of tissue handling, extraction efficiency, and satisfactory PCR results.

Key Words: DNA analysis; methacarn; microdissection; PCR; wax-embedded tissue.

1. Introduction

Tissue fixation and subsequent wax embedding are routinely employed for histological assessment because of the ease of handling tissues and subsequent staining as well as the good morphological preservation. Usually, formaldehyde-based fixatives are used for this purpose. However, with such crosslinking agents, there is limited performance in terms of the efficiency of extraction and quality of extracted RNA *(1–3)*, protein *(4,5)*, and genomic DNA *(6–9)*, with consequent difficulty in the analysis of microdissected, histologically defined tissue areas.

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Extraction efficiency and integrity of DNA are critical for the molecular analysis of microdissected cells. Recently, we have found that methacarn, a non-crosslinking protein-precipitating fixative *(10,11)*, meets critical criteria for analysis of RNA and proteins in wax-embedded tissue sections *(5)*. In the case of DNA extraction from formalin-fixed wax-embedded tissues, the extraction protocol usually requires proteinase K treatment with extended incubation periods *(12–14)*. On the other hand, we found that methacarn fixation allows high yields and amplification of long genomic DNA segments in wax-embedded tissue sections by a simple extraction procedure *(9,15)*.

Tissue staining is essential for cellular identification in practical molecular analysis using microdissection techniques *(13,16–21)*; therefore it is important to assess the effect of tissue staining on the performance of molecular analysis *(13,17,20)*. Furthermore, analysis of gene expression or mutation in immunophenotypically defined cells would be a versatile research technique *(19)*.

In this chapter, we detail the procedures for genomic DNA analysis in methacarn-fixed wax-embedded microdissected tissue specimens *(15)*, and illustrate its suitability in terms of target fragment size and the number of microdissected cells required for DNA analysis using cresyl-violet-stained sections. We also assess the effects of tissue staining with hematoxylin and eosin (H&E) or immunohistochemical stains on subsequent analysis of genomic DNA.

2. Materials

1. Methacarn, consisting of 60% (v/v) absolute methanol, 30% chloroform, and 10% glacial acetic acid.
2. Ethanol, 99.5% (v/v).
3. Shaker for tissue agitation.
4. Xylene, reagent-grade.
5. Tissue cassettes (Tissue-Tek® Cassette series; Sakura Finetek Japan Co. Ltd., Tokyo, Japan).
6. Tissue-embedding console system (Tissue-Tek® TEC™ 5; Sakura Finetek Japan).
7. Embedding molds (Base Molds for Tissue-Tek® Embedding Rings; Sakura Finetek Japan).
8. Embedding rings (Sakura Finetek Japan).
9. Wax (Sakura Finetek Japan).
10. Microtome.
11. Hematoxylin (Tissue-Tek® Hematoxylin 3G; Sakura Finetek Japan).
12. Eosin (Tissue-Tek® Eosin; Sakura Finetek Japan).
13. 0.1% Cresyl violet solution.
14. Primary antibodies for immunohistochemistry.
15. 1% Periodic acid solution.
16. Immunostaining kit (Vectastain Elite kit; Vector Laboratories Inc., Burlingame, CA).

17. 3,3'-Diaminobenzidine, tetrahydrochloride (DAB; Dojindo Laboratories; Kumamoto, Japan).
18. Hydrogen peroxide, 30% (w/w).
19. Casein (Merck, Darmstadt, Germany).
20. Microdissector (PALM Robot-MicroBeam equipment; Carl Zeiss Co., Ltd., Tokyo, Japan).
21. Polyethylene film for microdissection, 1.35 μm thick (PALM GmbH; Wolfratshausen, Germany).
22. Nail polish.
23. TaKaRa DEXPAT™ (Takara Bio Inc., Shiga, Japan).
24. Hoechst 33258 (Molecular Probe, Eugene, OR).
25. Fluorescence spectrophotometer.
26. Thermal cycler.
27. Oligonucleotide primers.
28. PCR buffer: 20 m*M* Tris-HCl (pH 8.4), 50 m*M* KCl, 0.2 m*M* dNTP, 1.5 m*M* $MgCl_2$.
29. PLATINUM *Taq* DNA polymerase (Invitrogen Corp, Carlsbad, CA).
30. Agarose gel electrophoresis equipment.
31. Ethidium bromide (10 mg/mL; Invitrogen).
32. Agarose and DNA sequencing equipment.
33. Autoclaved ultrapure water for preparation of solutions.

3. Methods

The methods described below outline (1) the preparation of methacarn-fixed wax-embedded tissue specimens, (2) tissue staining, (3) microdissection, (4) DNA extraction from microdissected cells, and (5) polymerase chain reaction (PCR).

3.1. Preparation of Methacarn-Fixed Wax-Embedded Tissue Specimens

Methacarn solution, which is easily prepared, should be freshly made and stored at 4°C before fixation *(22)* (*see* **Note 1**).

3.1.1. Fixation and Tissue Embedding

1. Trim tissues/organs to 3 mm in thickness if possible.
2. If necessary, each tissue can be placed on a piece of filter paper or into a tissue cassette (Sakura Finetek Japan) to support tissue shape.
3. Fix tissues with methacarn for 2 h at 4°C with gentle agitation using a shaker.
4. Dehydrate tissues three times for 1 h in fresh 99.5% ethanol at 4°C with agitation.
5. Trim tissues for embedding during **step 4** if necessary.
6. Immerse tissue in xylene for 1 h and then three times for 30 min at room temperature.
7. Immerse tissues in hot wax (60°C) three successive 1-h periods.
8. Embed tissue specimens in fresh wax using a tissue-embedding console system (Sakura Finetek Japan).
9. Store wax-embedded tissue blocks at 4°C until sectioning.

Table 1
Comparison of DNA Yields Between Unfixed or Ethanol-Fixed Frozen Tissues and Methacarn-Fixed Wax-Embedded Tissues[a]

Tissue condition	No. of sample	DNA yield (μg/mg wet tissue)
Unfixed frozen	5	1.05 ± 0.10
Ethanol-fixed frozen	5	1.15 ± 0.11
Methacarn-fixed wax-embedded	4	0.76 ± 0.06[b]

[a]Rat liver tissue was used as described previously in **ref. *15***. After dewaxing, extraction of DNA from methacarn-fixed wax-embedded small liver tissue blocks was performed by digestion with 500 μL of 10 m*M* Tris-HCl (pH 8.0), 150 m*M* NaCl, 10 m*M* ethylenediaminetetraacetic acid, 0.1% sodium dodecyl sulfate, and 1 U of proteinase K at 55°C for 2 h. The film was removed from the tube at this time point. Then 500 μL of Tris buffer-saturated phenol was added, mixed well, and centrifuged at 10,000*g* for 15 min. The supernatant was further extracted again with 500 μL of Tris-phenol/chloroform (1:1), and the separated aqueous portion after centrifugation was transferred to a new tube and treated with 0.5 U of RNase A at 37°C for 1 h. The solution was extracted with 500 μL of phenol/chloroform (1:1) and then treated with ether. Extracted DNA was precipitated by adding 1 μL of cold 99.5% ethanol, and after storing at –20°C overnight, centrifuged at 5000*g* for 5 min. The pellet was washed twice with 75% ethanol, dried, and resuspended in 10 μL of water. One milliliter of sample was used to measure DNA concentration by Hoechst 33258 and a fluorescence spectrophotometer. Extraction of DNA from methacarn-fixed wax-embedded small liver tissue blocks was performed after dewaxing. Frozen tissue blocks of unfixed or ethanol-fixed liver were directly subjected to DNA extraction, and dewaxed blocks of methacarn-fixed wax-embedded liver tissue were air-dried before extraction.

[b]Significantly different from unfixed frozen sample ($p < 0.01$ by ANOVA).

(Reproduced with permission from **ref. *15***.)

The yield and quality of extracted DNA are critical for the subsequent analysis of microdissected cells. DNA yield from ethanol-fixed frozen tissues is similar to that from unfixed frozen tissues (*see* **Table 1**) ***(15)***. On the other hand, DNA yield from methacarn-fixed wax-embedded tissue is slightly reduced, the mean value being about 70% of that from unfixed frozen tissues (**Table 1**). The integrity of extracted DNA from methacarn-fixed wax-embedded tissue is assessed by electrophoresis on 1.5% agarose gel (*see* **Fig. 1**). Although a slightly greater intensity at the top of the DNA smear is observed in unfixed or ethanol-fixed frozen samples, DNA in every case distributes within the high molecular weight range, suggesting good preservation of extracted DNA.

3.1.2. Preparation of Methacarn-Fixed Wax-Embedded Tissue Sections

1. Section at 5–10 μm in thickness using a microtome.
2. Stretch sections with slide warmer (Sakura Finetek Japan).
3. Mount stretched section onto a 1.35-μm thin polyethylene film (PALM GmbH) overlaid on a glass slide.

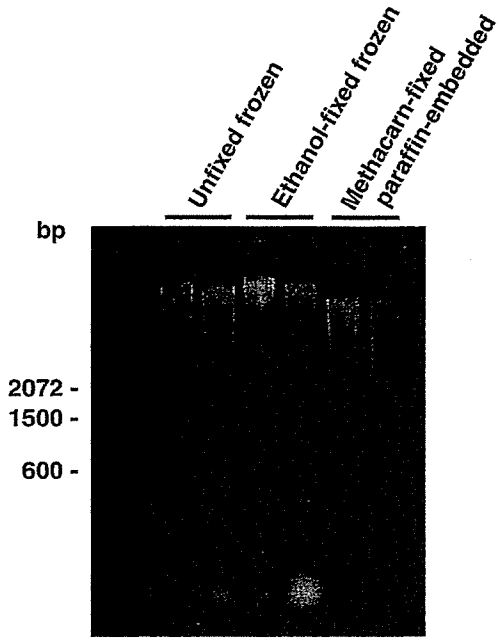

Fig. 1. Comparison of the integrity of DNA extracted from rat liver of unfixed or ethanol-fixed frozen tissue blocks and dewaxed methacarn-fixed tissue blocks. Tissue blocks were directly subjected to DNA extraction as described in the footnote of **Table 1**, and 2.0 μg of extracted DNA was subjected to electrophoresis in 1.5% agarose gel and stained with ethidium bromide as previously described *(15)*. (Reproduced with permission from **ref. 15**.)

4. Dry sections overnight at 37°C in an incubator.
5. Store sections at 4°C until use.

3.2. Tissue Staining

Dewax sections by immersing in xylene for 3 × 2 min followed by 99.5% ethanol for 2 × 2 min. Tissue sections can be stained with cresyl violet or H&E, or immunostained (*see* **Note 2**).

3.2.1. Cresyl Violet Staining

1. Dissolve 0.5 g cresyl violet in 500 mL water.
2. Add 8 drops of acetic acid.
3. If necessary, boil the solution until completely dissolved.
4. Filter the solution before using.
5. Immerse dewaxed sections briefly in water.

6. Incubate sections in cresyl violet solution for 20 min.
7. Wash sections once with 95% ethanol that contains 0.5% acetic acid, and then with 99.5% ethanol twice.
8. Air-dry.

3.2.2. Hematoxylin and Eosin Staining

1. Immerse dewaxed sections briefly in water.
2. Immerse sections in hematoxylin solution (Tissue-Tek® Hematoxylin 3G) for 10 s.
3. Wash sections briefly with water.
4. Immerse sections with eosin solution (Tissue-Tek® Eosin) for 10 s.
5. Wash sections briefly with 99.5% ethanol.
6. Air-dry.

3.2.3. Immunostaining

1. Treat dewaxed sections with 1% periodic acid solution for 10 min.
2. Wash sections briefly with water and 1X phosphate-buffered saline (PBS; pH 7.4).
3. Block nonspecific binding sites with 0.5% casein in PBS for 30 min.
4. Incubate with primary antibody of appropriate dilution for 2 h.
5. Wash sections with PBS for 5 min × 3.
6. Incubate sections with biotin-labeled secondary antibody.
7. Repeat **step 5**.
8. Incubate sections with avidin-biotin complex utilizing Vectastain Elite kit (Vector Laboratories).
9. Repeat **step 5**.
10. Visualize immunoreaction using the avidin/biotin system with 0.004% hydrogen peroxide as substrate and DAB as chromogen.
11. Rinse sections with water.
12. Perform nuclear staining with hematoxylin if desired (*see* **Subheading 3.2.2.**).
13. Air-dry.

Tissue staining can affect the yield and quality of extracted DNA with methacarn-fixed wax-embedded tissue sections (*see* **Table 2** and **Fig. 2**) ***(15)***. **Table 2** shows the DNA yield from methacarn-fixed wax-embedded tissue sec-

Fig. 2. (*opposite page*) Integrity of DNA extracted from stained sections of methacarn-fixed wax-embedded tissue. Liver of a rat treated with thioacetamide at the promotion stage in the two-stage hepatocarcinogenesis model ***(23,24)*** was used. Tissue blocks were trimmed to obtain sections of 100 mm^2 in area before sectioning, and sectioned at 10 μm in thickness. Serial sections were randomized and mounted onto polyethylene film overlaid on a glass slide. Dewaxed sections were either unstained, stained with H&E, or immunostained with glutathione-*S*-transferase placental form

Table 2
Effect of Staining on Yield of Extracted DNA From Methacarn-Fixed Rat Liver Wax-Embedded Tissue Sections[a]

Tissue condition	No. of samples	Yield of DNA (ng/100 mm^2 area)[b]	Ratio of unstained section (%)
Unstained	10	2705.1 ± 853.4	100
H&E-stained	5	2687.4 ± 632.4	99.3
Immunostained[c]	13	314.5 ±85.2[d]	11.6

[a]Liver of a rat treated with thioacetamide at the promotion stage in the two-stage hepatocarcinogenesis model *(23,24)* was used as described previously in **ref. *15***.

[b]Tissue blocks were trimmed to 100 mm^2 before sectioning, and sectioned at 10 µm. DNA extraction and estimation of its concentration were performed according to the methods described in **Table 1**.

[c]Sections were immunostained with GST-P.

[d]Significantly different from the unstained and H&E-stained samples ($p < 0.0001$ by ANOVA).

(Reproduced with permission from **ref. *15***.)

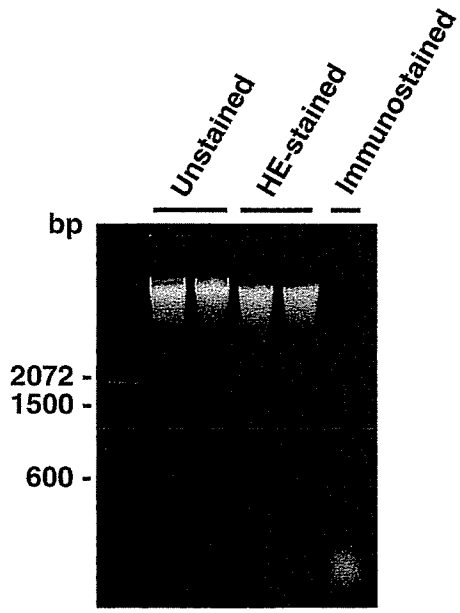

Fig. 2. (*continued*) (GST-P) (*see* **Subheadings 3.2.2.** and **3.2.3.**). One µg of extracted DNA from whole tissue section was subjected to electrophoresis in 1.5% agarose gel and stained with ethidium bromide as described previously *(15)*. (Reproduced with permission from **ref. *15***.)

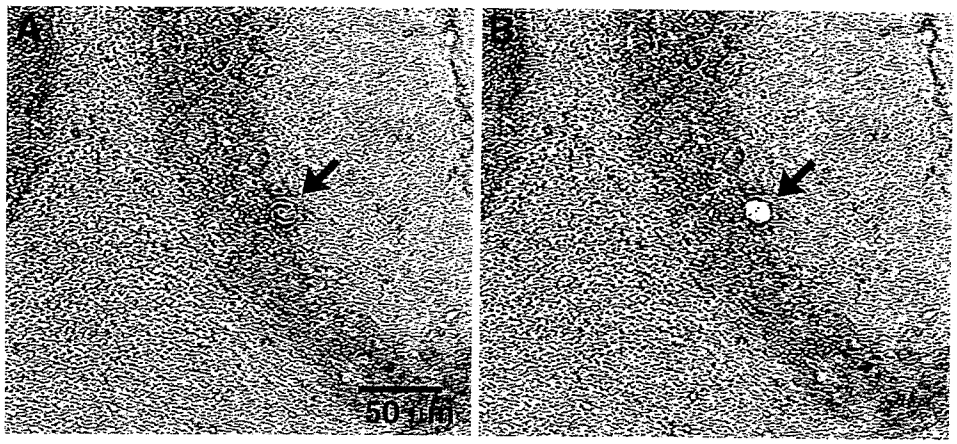

Fig. 3. Microdissection of single Purkinje cells from cresyl violet-stained rat cerebellum section as described previously in **ref. *15***. **(A)** Wax-embedded rat cerebellum sectioned at 10 µm in thickness was mounted on a film, dewaxed, and stained with cresyl violet (*see* **Subheading 3.2.1.**). A single Purkinje cell was selected and microdissected from the surrounding tissue with a laser beam (arrow). **(B)** The Purkinje cell has been cut out and catapulted by laser pressure (arrow). (Reproduced with permission from **ref. *15***.)

tions after tissue staining. The yield recovered from H&E-stained sections is similar to that from unstained sections. Immunostained tissue sections, on the other hand, result in very low DNA yield, values being 12% those of unstained sections. **Fig. 2** shows the integrity of DNA extracted from stained sections as visualized by electrophoresis on 1.5% agarose gel. DNA from unstained sections distributes mainly within the high molecular weight range. Similar to the unstained tissue section, H&E-stained sections show good preservation of the extracted DNA. As compared to unstained and H&E-stained cases, DNA extracted after immunostaining shows significant degradation of the DNA with small DNA fragments of approx 100 bp size (*see* **Note 3**).

3.3. Microdissection

Microdissection is performed with PALM Robot-MicroBeam equipment (Carl Zeiss Co., Ltd.) as described previously (*see* **Note 4**) *(25)*. Briefly, the film with the attached specimen is mounted in reverse (film side up) onto a new cover slip (26 × 76 mm) by adhering the film to the cover slip with nail polish. The specimens are then subjected to Robot-MicroBeam dissection by laser beam and the selected cells are catapulted by laser pressure into mineral oil-coated PCR tube caps (**Fig. 3**) *(15)*. In case of large specimens (circle areas of 150–200 µm in radius or square areas larger than 60 × 60 µm), the excised

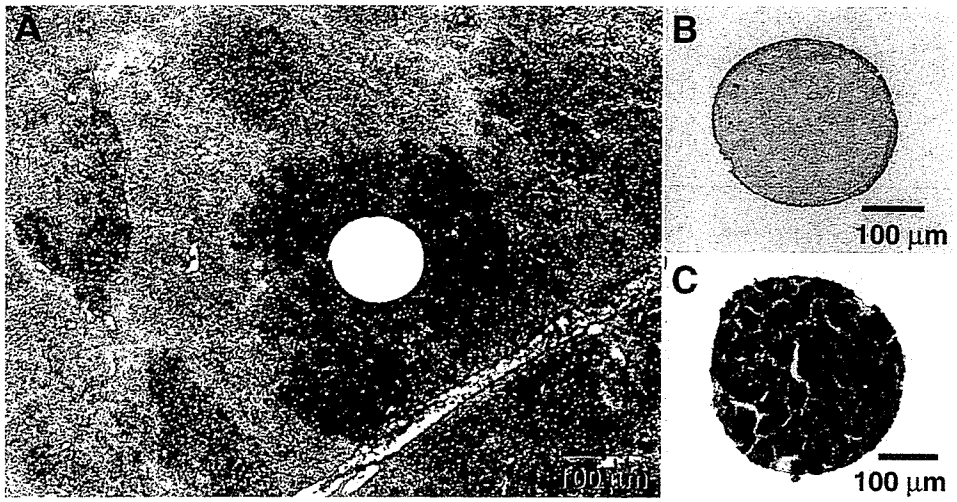

Fig. 4. Microdissection using a liver section immunostained with GST-P from a thioacetamide-treated rat in the two-stage hepatocarcinogenesis model as described previously in **ref. *15***. (**A**) A liver section (10-μm thick) mounted on polyethylene film was immunostained with GST-P (*see* **Subheading 3.2.3.**) and a circle area of 150 μm radius within a GST-P-positive focus was microdissected. (**B**) Removed GST-P-positive cellular area from the section shown in **Fig. 4A**. (**C**) Identical portions of circle area in GST-P-positive foci were microdissected from H&E-stained adjacent section. A mean of 150 cells were contained in the circle area ($n = 5$). (Reproduced with permission from **ref. *15***.)

cells can be picked up with a thin needle tip (**Fig. 4**) ***(15)***. Transfer of microdissected specimen on the cap of a PCR tube should be verified under a microscope.

3.4. DNA Extraction From Microdissected Cells

Microdissected cells or tissue areas on PCR tube caps are subjected to extraction with 4 μL of TaKaRa DEXPAT™ (*see* **Note 5**) at 95°C for 10 min, and the entire extracts are used as a template for PCR by adding to the master mix of total 50 μL directly as described in **Subheadings 3.5.1.** and **3.5.2.** ***(15)***. In the case of a large cellular area such as 1 × 1 mm area, tissue specimens are extracted with 40 μL of DEXPAT.

3.5. PCR

PCR is the major tool for analysis of genomic DNA; cycle numbers should be minimized to avoid amplification-derived DNA-polymerization errors. Hot-start PCR of the genomic sequence of the gene of interest is performed with

PLATINUM *Taq* DNA polymerase in a 50-µL total reaction volume *(15)*. If nested PCR is intended, 1 µL of the first PCR product is used as a template in a 20-µL total volume (*see* **Note 6**).

3.5.1. Amplification by Nested PCR

1. Aliquot 4 µL of extracted DNA and mix with PCR reaction mixture contained 20 m*M* Tris-HCl (pH 8.4), 50 m*M* KCl, 0.2 m*M* dNTP, 1.5 m*M* $MgCl_2$, 0.2 µ*M* each primer, and 2.5 U of *Taq* DNA polymerase in a 50-µL total volume.
2. Perform first-step PCR of 20–35 cycles.
3. Aliquot 1 µL of the first PCR product and mix with PCR reaction mixture with 1 U of PLATINUM *Taq* DNA polymerase in a 20-µL total volume.
4. Perform second-step PCR of 20–35 cycles.
5. Aliquot 8 µL of the PCR product and run agarose gel electrophoresis to identify the amplified target fragment (*see* **Fig. 5**).
6. Aliquot 10 µL of the PCR product for direct sequencing.

By nested PCR, the 522-bp DNA fragment of the α_{2u}-globulin gene is successfully amplified in 20% of the PCR attempts of single Purkinje cells with a total of 70 PCR cycles from cresyl violet-stained rat cerebellum sections (*see* **Table 3**) *(15)*. Similar, but less effective, amplification can be obtained with microdissected areas of hippocampal CA1 region, in which a successful detection is obtained in 15% of 20 × 20 µm samples (corresponding to 2.4 cells). The frequency of PCR detection increases with the area microdissected, but does not reach 100% even in a 60 × 60 µm area.

3.5.2. Amplification by Single-Step PCR

1. Aliquot 4 µL of extracted DNA and mix with PCR reaction mixture as described in **Subheading 3.5.1.** and 2.5 U of *Taq* DNA polymerase in a 50-µL total volume.
2. Perform PCR of 35 cycles.
3. Aliquot 8 µL of the PCR product and run agarose gel electrophoresis to identify the amplified target fragment.
4. Aliquot 10 µL of the PCR product for direct sequencing.

In the PALM system, either a rectangle or a circle of any size can be microdissected in automated mode. In H&E-stained rat liver sections as described in **Fig. 4**, a 522-bp fragment can be amplified by single-step PCR of 35 cycles with both 150- and 200-µm-radius samples after DEXPAT extraction, although the amplification of 969-bp fragments is unsuccessful even with 200-µm-radius samples (*see* **Table 4**) *(15)*. Liver samples of 150- and 200-µm-radius areas in this case contain 150 and 270 cells, respectively. In immunostained tissue, a weak 522-bp band can be amplified only with 200-µm-radius samples. In the case of 150-µm-radius samples, only a 184-bp fragment can be amplified.

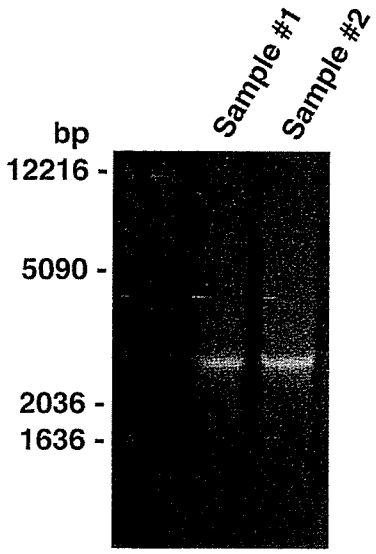

Fig. 5. Nested PCR results for the α_{2u}-globulin genomic sequence (Accession no. M24108 in GenBank/EMBL Data Bank), sized 2.8 kb, with DNA extracted from methacarn-fixed wax-embedded rat cerebral cortex as described previously *(15)*. From 10-μm thick, cresyl violet-stained brain sections, 1 × 1 mm areas of cerebral cortex were microdissected and extracted with 40 μL of DEXPAT to extract DNA. Four-μL aliquots of cell extracts were directly applied for the first PCR reaction with upstream-outside primer, 5'-ACGGATCCAG GCTTCAAGTT CCGTATTA-3' and downstream primer for the 2954-bp fragment, 5'-TGAAATCCTG AGACTAAGCT-3'. With 1 μL of the first PCR product, second-step PCR was performed to amplify a 2.8-kb fraction with a combination of upstream-inside primer, 5'-AAAGTTAAAT GGAATCAGAA-3', and the downstream primer used for the 2954-bp fragment in the first PCR. Nested PCR in a 20-μL total volume was performed using 1 μL of the first PCR product as a template. This figure shows results of two different samples. (Reproduced with permission from **ref. *15***.)

4. Notes

1. Exposure of tissues to saline prior to fixation may cause a severe tissue shrinkage artifact *(10)*. Glassware for the preparation of methacarn should be autoclaved before use. Do not use disposable plasticware that can be damaged by chloroform for the preparation and/or storage of methacarn solution. For fixation, the ratio of the fixative volume to tissue volume should be 20:1–30:1. If necessary, tissue processing can be stopped at the step of ethanol dehydration, and tissue blocks can be kept in ethanol at 4°C for several days after fixation.
2. Immersion of the tissue section in aqueous solution for a long time may increase the risk of degradation of DNA *(15)*. If nuclear staining is intended, the staining

Table 3
Detection of 522-bp DNA Fragment From Microdissected Single Cells or Cellular Areas by Nested PCR[a]

Cell or cellular area	Microdissected	No. of samples	PCR-detection (%)
Purkinje cell	single cell	15	20
Hippocampus,	20 × 20 μm (2.4 cells)[b]	26	15
CA1 region	40 × 40 μm (9.5 cells)[b]	15	67
	60 × 60 μm (21.3 cells)[b]	15	87
Cerebral cortex[c]	1 × 1 mm	24	100

[a]Rat α_{2u}-globulin gene was used for PCR as described previously in **ref. *15***. First-step PCR was performed to amplify a 969-bp fragment with an upstream-outside primer, 5'-ACGGATCCAG GCTTCAAGTT CCGTATTA-3', and a downstream primer, 5'-CGTCATCTGT GGAGGAAATT-3'. With 1 μL of the first PCR product, second-step was performed to amplify a 522-bp fragment with an upstream-inside primer, 5'-AAAGTTAAAT GGAATCAGAA-3', and a downstream-inside primer, 5'-TAAGTCCGTC TCACATGGCT-3'.

[b]Mean cell number in a 60 × 60 μm area (n = 18) was estimated with the aid of an objective micrometer, and the mean cell number in each square area was calculated.

[c]Extract from 1 × 1 mm area of cerebral cortex was further diluted with DEXPAT to adjust the concentration of template to correspond to a 60 × 60 μm area for first-step PCR.

(Reproduced with permission from **ref. *15***.)

solution should be autoclaved or filtrated if possible. Methacarn-fixed tissue sections can be stained more quickly than formalin-fixed tissue sections, and therefore the time of the histological staining procedure can be reduced.

3. Immunostaining of methacarn-fixed wax-embedded tissue results in a substantial decrease in DNA yield, in particular the loss of high molecular weight DNA. There is progressive decrease in DNA yield in proportion to the length of the immunostaining process *(15)*.
4. There are two major techniques for microdissection utilizing the precision of lasers. One technique is laser microbeam microdissection as employed in our laboratory; this system is based on a pulsed UV laser with a small beam focus to cut out areas or cells of interest by photoablation of adjacent tissue. Another technique is laser capture microdissection, which uses a low-energy infrared laser pulse to capture the targeted cells by focal melting of the thermoplastic membrane through laser activation. Advantages and disadvantages of these systems are described elsewhere *(26)*.
5. TaKaRa DEXPAT is a reagent originally designed for one-step extraction of DNA from wax-embedded tissue fixed with 10% formalin. DEXPAT is designed to optimize DNA extraction from wax embedded tissue; it utilizes ion exchange resin and detergents, and DNA is extracted in the supernatant. We use only the detergent component.With methacarn-fixed wax-embedded tissues, the time for the preparation of PCR-ready DNA from wax-embedded tissue is dramatically reduced from 2 to 3 d required for a conventional method for formalin-fixed tissues to 25 min.

Table 4
Detection of Genomic DNA Fragment by Single-Step PCR in H&E-Stained or Immunostained Tissue Areas From Methacarn-Fixed Rat Liver Wax-Embedded Tissue Sections[a]

Stain	Tissue area (μm in radius)	Fragment size (bp)	Positive detection
H&E-stained	200	184[b]	8/8 (100%)
	200	522[c]	9/9 (100%)
	200	969[c]	0/8 (0%)
	150	184	5/5 (100%)
	150	522	5/5 (100%)
Immunostained[d]	200	184	5/5 (100%)
	200	522	5/5 (100%)
	150	184	10/10 (100%)
	150	522	0/5 (0%)

[a]Liver of a rat treated with thioacetamide at the promotion stage in the two-stage hepatocarcinogenesis model was used as described previously in **ref. *15***. Circle areas of 150 or 200 μm in radius were microdissected and solubilized with 4 μL of DEXPAT solution in PCR tubes at 95°C for 10 min and whole extracts were subjected to PCR directly. PCR with 50 μL reaction volume was performed to amplify 184-, 522-, and 969-bp fragments with the same cycle parameters of 95°C for 2 min, 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 30 s, and final extension at 72°C for 7 min. Eight microliters of PCR product was applied to 2.0% agarose gel electrophoresis.

[b]Rat GST-P gene (Accession no. L29427 in GenBank/EMBL Data Bank) was amplified using upstream primer, 5'-GGAGCAGGAC CCAAAAATGA-3', and downstream primer, 5'-GCA GACGAAT AAAGGCCCCA-3'.

[c]Rat α_{2u}-globulin gene was used for amplification. Primer pairs for each DNA fragment were similar to those described in the footnote of **Table 3**.

[d]Sections were immunostained with GST-P.

(Reproduced with permission from **ref. *15***.)

6. To amplify target fragment sizes smaller than 1 kb, PCR was performed with cycle parameters of 95°C for 5 min, 35 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 30 s. The extension time for 2, 3, and 4 kb is 1.5, 2.5, and 3.5 min respectively. Although the source of cells and the detection system are different from those in the present study, similar performance was obtained when DNA from 25 cells of alcohol-fixed cytology specimens was used in the multiplex PCR *(27)*.

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